REMARKS

Claims 21-26 are pending. The specification is objected to. Claims 21-26 are rejected. The specification, in particular the title, is amended to be more descriptive of the present invention. Claims 21-25 are amended. Support for amended Claims 21-25 can be found, e.g., in Claims 7, 8, 9, and 11, as originally filed. Amended Claim 24 also finds support on, e.g., page 62, lines 19-28, of the specification. Claim 26 is cancelled, without prejudice. Claim 27 is new and is supported by Claim 7, as originally filed.

Applicant believes that no new matter is added by way of amendment.

Applicant notes that the Examiner withdrew the Restriction Requirement between Groups III and IV.

The Examiner alleges that the relevance of references AE-AG and AL-AN cannot be assessed. References AE-AG are relevant because sequences disclosed therein are aligned with the DCRS9 of the present invention (Table 6, pages 39-40, of the specification).

I. Objections to the Specification.

The Examiner objected to the title of the invention as not being descriptive of the present invention. Applicant has amended the title accordingly. Withdrawal of this objection is respectfully requested.

II. Rejections of Claims 21-26 under 35 U.S.C. §101.

Claims 21-26 are rejected under 35 U.S.C. §101, and on the basis of a lack of support by either a credible, specific, and substantial utility, or a well established utility. Applicant submits that the rejection of Claim 26 is moot, in view of the cancellation of this claim.

The Examiner agrees that the present invention is a "cytokine receptor protein," but alleges that function cannot be predicted based solely on homology (page 3, lines 23-25, of Office Action). Applicant respectfully disagrees that function is based solely

on homology. The Examiner's initial burden is to establish that it is more likely than not that a person of ordinary skill would not consider that any utility asserted by the Applicant would be credible, specific, and substantial. According to the MPEP, "[o]nly where the totality of the record continues to show that the asserted utility is not specific, substantial, and credible, should a rejection based on lack of utility be maintained. If the record as a whole would make it more likely than not that the asserted utility for the claimed invention would be considered credible by a person of ordinary skill in the art, the Office cannot maintain the rejection. *In re Rinehart*, 531 F.2d 1048, 1052; 189 USPQ 143, 147 (CCPA 1976)" (§2107.02 VI MPEP, August 2001).

The totality of the record includes not only the above-mentioned homology data, but also data on helminth challenge and DCRS9 expression (pages 80-81 of the specification), additional expression data for DCRS9 and expression data for the DCRS9's ligand (a.k.a. IL-71) (Declaration under 37 C.F.R. §1.132 by Daniel M. Gorman) (enclosed), and data on IL-71's's stimulation of neutrophil infiltration in lungs (Hurst, et al. (2002) *J. Immunol.* 169:443-453) (enclosed).

The specification asserts that the present invention is involved in innate immunity, e.g., "[t]he cytokine receptor-like proteins will . . . result in modulation of . . . innate immunity response. . ." and that "[t]he cytokine receptors . . . and antibodies, should be useful in the treatment of . . . innate immunity . . ." (page 43, lines 25-27; page 65, lines 10-11, of specification). Innate immunity relates to immune response in the earliest states of infection, e.g., before the later adaptive response involving lymphocytes, see, e.g., Roitt, et al. (1993) *Immunology, 3rd ed.*, Mosby, St. Louis, p. 1.2. Moreover, data in the specification correlates the present invention with an innate immunity response to a helminth, *Ascaris*. These data disclose that expression of the present invention is transiently elevated in lung at 4 hours after initial exposure to *Ascaris*, i.e., *Ascaris* challenge (pages 80-81, of specification).

At the time of filing, it was well established that the lung shows an innate response to *Ascaris* challenge, where this response includes transient responses, see, e.g., Abraham, et al. (1999) *Am. J. Respir. Crit. Care Med.* 159:1205-1214 (enclosed);

Jones, et al. (1998) *Can. J. Physiol. Pharmacol.* 76:210-217 (enclosed); Wright, et al. (1999) *J. Pharmacol. Exp. Therapeutics* 289:1007-1014; D'Brot, et al. (1989) *Am. Rev. Respir. Dis.* 139:915-920. Thus, response to *Ascaris* challenge taking the form of a transient host response was accepted by those skilled in the art. The data of page 80-81 of the specification relating to response over a period of time are consistent with the time courses disclosed in, e.g., Abraham, supra, and Jones, supra.

According to the MPEP, "new evidence submitted in an affidavit or declaration under 37 CFR §1.132, or in a printed publication" is part of the totality of the record that can be used to rebut a rejection under 35 U.S.C. §101 (§2107.02 VI MPEP, August 2001).

The Declaration under 37 C.F.R. §1.132 by inventor Daniel M. Gorman (enclosed), confirms that expression of the DCRS9 of the present invention increases after helminth challenge, as disclosed in the specification. The Declaration describes data generated after the priority date of the present invention that show a significant increase in expression of DCRS9 in mouse lung, after helminth challenge. The Declaration also establishes that the ligand of DCRS9 to be specifically identified as IL-71 (a.k.a. IL-17C), and expression of IL-71 increases after helminth challenge.

The Declaration is further supported by the Hurst, supra, reference which discloses that IL-17C stimulates an increase in neutrophils in the lungs (page 446, column 1, of Hurst, supra). Neutrophils are specifically associated with innate responses, see, e.g., Abbas, et al. (2000) *Cellular and Molecular Immunology* 4th ed., W.B. Saunders Co., Phila., PA, pp. 4-5 (enclosed); Roitt, et al. (1993) *Immunology*, 3rd ed., Mosby, St. Louis, MO, pp. 1.1-1.2) (enclosed). Taken together, the data on pages 80-81 of the specification, the Declaration, and Hurst, supra, confirm Applicant's asserted utility of the present invention in innate responses. Applicant therefore submits that one of ordinary skill would more likely believe the asserted utility for the claimed invention, as at least a marker for innate immunity, to be credible, substantial, and specific. Applicant also submits that new Claim 27 does not contain any basis for rejection under 35 U.S.C. §101.

Applicant believes the rejection of Claims 21-26 under 35 U.S.C. §101 to be overcome. Withdrawal of this rejection is respectfully requested.

III. Rejections of Claims 21-26 under 35 U.S.C. §112, First Paragraph.

The Examiner rejected Claims 21-26 under 35 U.S.C. §112, first paragraph (page 5, lines 19-27, of Office Action). Applicant submits that the rejection of Claim 26 is moot, in view of the cancellation of this claim. The basis for the rejection is the alleged lack of a credible, substantial, and specific utility, or a well-established utility, thereby not teaching the skilled artisan how to make and use the present invention. As noted above, Applicant has submitted rebuttal arguments, a Declaration, and published articles establishing that the present invention has a credible, specific, and substantial utility, and that therefore the skilled artisan would know how to make and use the present invention. Applicant submits that new Claim 27 does not contain any basis for rejection under 35 U.S.C. §112, first paragraph.

Applicant believes that the rejection of Claims 21-26 under 35 U.S.C. §112, first paragraph, is overcome by the foregoing. Withdrawal of this rejection is respectfully requested.

IV. Rejections of Claims 22-26 under 35 U.S.C. §112, Second Paragraph.

The Examiner rejected Claims 22-26 under 35 U.S.C. §112, second paragraph, on the basis of indefiniteness. Applicant submits that the rejection of Claim 26 is moot, in view of the cancellation of this claim.

The Examiner alleges that Claim 22 is indefinite for reciting "further comprising," which indicates an additional active ingredient. Applicant believes that amended Claim 22 does not indicate an additional ingredient.

Furthermore, the Examiner alleges that Claim 23 is confusing for reciting a composition comprising a composition. Applicant has amended Claim 23 as suggested by the Examiner.

Moreover, the Examiner alleges that Claim 24 must explicitly state a relation between two or more elements, and that instructional material is not given weight. Applicant believes the amended Claim 24 recites a relation between two elements. Applicant also submits that new Claim 27 does not contain any basis for rejections under 35 U.S.C. §112, second paragraph.

In view of the foregoing, Applicant submits that the rejection of Claims 22-26 under 35 U.S.C. §112, second paragraph, is overcome. Withdrawal of this rejection is respectfully requested.

V. Rejections of Claims 21-23 and 25-26 under 35 U.S.C. §102(b).

The Examiner rejected Claims 21-23 and 25-26 under 35 U.S.C. §102(b), as anticipated by Koskinen, et al. (1998) *J. Immunol.* 160:4943-4950, on the basis that the antibody of Koskinen, supra, would be expected to bind to an "antigenic fragment" of SEQ ID NO:12 (page 7, line 11, of Office Action). Applicant submits that the rejection of Claim 26 is moot, in view of the cancellation of this claim.

Amended Claim 21 no longer recites "an antigenic fragment" of SEQ ID NO:12, thus Koskinen does not anticipate the present invention, nor do remaining Claims 22-25 and 27, which depend directly or indirectly from Claim 21.

Moreover, the Examiner admits that the Koskinen reference teaches an antibody that "specifically" binds to CD5 (page 7, lines 6-7, of Office Action). Koskinen teaches that specificity of antibody binding is determined by antibody cross-reactivity assays, e.g., see page 4945, column 2, and Fig. 3D, of Koskinen. The Koskinen definition is consistent with the definition set forth in the specification, where, e.g., specific binding is defined in terms of being "selected to have low cross-reactivity" against other proteins (page 60, lines 15-16 and 35-37; and page 61, lines 1-14, of specification). Applicant also submits that new Claim 27 does not contain any basis for rejections under 35 U.S.C. §102(b).

In view of the foregoing, Applicant submits that the rejection of Claims 21-23 and 25-26 under 35 U.S.C. §102(b) is overcome. Withdrawal of this rejection is respectfully requested.

VI. Rejections of Claims 21-23 and 25 under 35 U.S.C. §102(e).

The Examiner rejected Claims 21-23 and 25 under 35 U.S.C. §102(e), as anticipated by U.S. Pat. No. 6,150,502 issued to Strachan. The Examiner alleges that SEQ ID NO:303 of Strachan shares 100% sequence identity with amino acids 1-44, 184-351, 356-421, and 423-657, of SEQ ID NO:12.

Applicant notes that the cited fragments do not have 100% sequence identity with fragments of the present invention (see Appendix I). Furthermore, amended Claim 21 does not recite antigenic fragments, nor do Claims 22, 23, and 25, which depend from Claim 21.

Moreover, Applicant points out that Claim 21 recites "specifically binds," a term defined, e.g., on page 60, lines 15-16 and 35-37; and page 61, lines 1-14, of specification. For example, specific binding is defined in terms of being "selected to have low cross-reactivity against other cytokine receptor family members." (page 60, lines 15-16, of specification). "Low cross-reactivity against other cytokine receptor family members" excludes the Strachan antibodies, because Strachan identifies its disclosed polypeptides as, e.g., "cytokines or their cognate receptors." (column 9, lines 27-28, of Strachan). Further, the specification provides a protocol for determining specific binding, where the protocol is a competition binding assay involving immobilized antigen and an added soluble, second protein (page 60, lines 35-37, to page 61, lines 1-2, of specification). This protocol for "specific binding" also excludes the Strachan antibodies from anticipating the antibodies of the present invention. Thus, Strachan fails to anticipate the present invention of Claims 21-23 and 25. Applicant also submits that new Claim 27 does not contain any basis for rejections under 35 U.S.C. §102(e).

In view of the foregoing, Applicant submits that the rejection of Claims 21-23 and 25 under 35 U.S.C. §102(e) is overcome. Withdrawal of this rejection is respectfully requested.

VII. Rejections of Claims 22-24 under 35 U.S.C. §102(a).

The Examiner rejected Claims 22-24 and 30-31 under 35 U.S.C. §102(a), as being anticipated by WO 99/55865-A1, on the same basis as noted above. Applicant wishes to point out that Claims 30-31 are not currently pending.

As noted above, the fragments of WO 99/55865-A1 do not share 100% sequence identity, and the antibody of the present invention would not "specifically bind" to the cited polypeptides. Thus, the cited reference fails to anticipate the present invention. Applicant also submits that new Claim 27 does not contain any basis for rejections under 35 U.S.C. §102(a).

In view of the foregoing, Applicant submits that the rejection of Claims 22-24 under 35 U.S.C. §102(a) is overcome. Withdrawal of this rejection is respectfully requested.

VIII. Rejections of Claim 24 under 35 U.S.C. §103(a).

The Examiner rejected Claim 24 under 35 U.S.C. §103(a), as obvious in view of Koskinen, supra, or the Strachan patent, in combination with the skill in the art (page 8, lines 10-24, of Office Action).

Applicant submits that Koskinen and Strachan have both been removed as prior art as noted above. Applicant also believes that the skill in the art at the time of filing cannot cure the deficiencies of Koskinen or Strachan. Applicant also submits that amended Claim 24 and new Claim 27 do not contain any basis for rejections under 35 U.S.C. §103.

In view of the foregoing, Applicant believes that the rejection of Claim 24 under 35 U.S.C. §103 is overcome. Withdrawal of this rejection is respectfully requested.

Conclusion

Applicant's current response is believed to be a complete reply to all the outstanding issues of the latest Office Action. Further, the present response is a bona fide effort to place the application in condition for allowance or in better form for appeal. Accordingly, Applicant respectfully requests reconsideration and passage of the amended claims to allowance at the earliest possible convenience.

Applicant believes that no additional fees are due with this communication. Should this not be the case, the Commissioner is hereby authorized to debit any charges or refund any overpayments to DNAX Deposit Account No. 04-1239.

If the Examiner believes that a telephonic conference would aid the prosecution of this case in any way, please call the undersigned.

Respectfully submitted,

Dated: September 26, 2003

By: Tom Brody
Tom Brody, Patent Agent
for Applicant
Reg. No. 46,433

DNAX Research, Inc.
901 California Avenue
Palo Alto, California 94304-1104
Tel: 650-496-6400
Fax: 650-496-1200

Enclosed:

1. Declaration of Daniel M. Gorman (5 pages).
2. Abbas, et al. (2000) *Cellular and Molecular Immunology* 4th ed., W.B. Saunders Co., Phila., PA, pp. 4-5 (4 pages).
3. Roitt, et al. (1993) *Immunology*, 3rd ed., Mosby, St. Louis, MO, pp. 1.1-1.2 (4 pages).
4. Abraham, et al. (1999) *Am. J. Respir. Crit. Care Med.* 159:1205-1214 (10 pages).
5. Jones, et al. (1998) *Can. J. Physiol. Pharmacol.* 76:210-217 (8 pages).
6. Hurst, et al. (2002) *J. Immunol.* 169:443-453 (11 pages).

APPENDIX I

Four regions of alleged 100% sequence identity (bolded and underlined) between SEQ ID NO:12 of the present invention and SEQ ID NO:303 of U.S. Pat. No. 6,150,502.

```
                                              1                                                50
Human DCRS9 SEQ ID NO:12              (1)  MGSSRLAALLLPLLLIVIDLSDSAGIGFRHLPHWNTRCPLASHTEVLPIS
     SEQ ID NO:303 from US 6,150,502  (1)  MGSPRLAALLLSLPLLLIGLAVSARVACPCLRSWTSHCLLAYRVDKRFAG
                           Consensus  (1)  MGS RLAALLL L LILI LA SA IA    L  W S C LA    D

                                              51                                              100
Human DCRS9 SEQ ID NO:12             (51)  LAAPGGPSSPQSLGVCESGIVPAVCASICCQVAQVFNGASSTSWCRNPKS
     SEQ ID NO:303 from US 6,150,502 (51)  LQWGWFP------------------LLVR--KSKS-------P--PKF
                           Consensus (51)  L     P                     I                  PK

                                              101                                             150
Human DCRS9 SEQ ID NO:12            (101)  LPHSSSIGDTRCQHLLRGSCCLVVTCLRRAITFPSPPQTSPTRDFALKGP
     SEQ ID NO:303 from US 6,150,502 (70)  EDYWRHRTPASFQRKLLGSPSLSEESHRISIPS--------S--AISHR-
                           Consensus (101)  H          Q  L GS  L      R AI           S

                                              151                                             200
Human DCRS9 SEQ ID NO:12            (151)  NLRIQRHGKVFPDWTHKGMEVGTGYNRRWVQLSGGPEFSFDLLPEARAIR
     SEQ ID NO:303 from US 6,150,502 (109)  GQRTKRAQPSAAEGREHLPEAGS-------QKCGGPEFSFDLLPEVQAVR
                           Consensus (151)   R  R        D        E GS      Q  GGPEFSFDLLPE  AIR

                                              201                                             250
Human DCRS9 SEQ ID NO:12            (201)  VTISSGPEVSVRLCHQWALECEELSSPYDVQKIVSGGHTVELPYEFLLPC
     SEQ ID NO:303 from US 6,150,502 (152)  VTIPAGPKASVRLCYQWALECEDLSSPFDTQKIVSGGHTVDLPYEFLLPC
                           Consensus (201)  VTI AGP  SVRLCHQWALECEDLSSPFD QKIVSGGHTVDLPYEFLLPC

                                              251                                             300
Human DCRS9 SEQ ID NO:12            (251)  LCIEASYLQEDTVRRKKCPFQSWPEAYGSDFWKSVHFTDYSQHTQMVMAL
     SEQ ID NO:303 from US 6,150,502 (202)  MCIEASYLQEDTVRRKKCPFQSWPEAYGSDFWQSIRFTDYSQHNQMVMAL
                           Consensus (251)  LCIEASYLQEDTVRRKKCPFQSWPEAYGSDFW SI FTDYSQH QMVMAL
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                                                  301                                               350
Human DCRS9 SEQ ID NO:12                   (301)  TLRCPLKLEAALCQRHDWHTLCKDLPNATARESDGWYVLEKVDLHPQLCF
     SEQ ID NO:303 from US 6,150,502       (252)  TLRCPLKLEASLCWRQDPLTPCETLPNATAQESEGWYILENVDLHPQLCF
                           Consensus       (301)  TLRCPLKLEAALC R D  T C  LPNATA ESDGWYILE VDLHPQLCF

                                                  351                                               400
Human DCRS9 SEQ ID NO:12                   (351)  KVQPWFSFGNSSHVECPHQTGSLTSWNVSMDTQAQQLILHFSSRMHATFS
     SEQ ID NO:303 from US 6,150,502       (302)  K----FSFENSSHVECPHQSGSLPSWTVSMDTQAQQLTLHFSSRTYATFS
                           Consensus       (351)  K    FSF NSSHVECPHQSGSL SW VSMDTQAQQL LHFSSR HATFS

                                                  401                                               450
Human DCRS9 SEQ ID NO:12                   (401)  AAWSLPGLGQDTLVPPVYTVSQ-----------------------VWRSD
     SEQ ID NO:303 from US 6,150,502       (348)  AAWSDPGLGPDTPMPPVYSISQTQGSVPVTLDLIIPFLRQENCILVWRSD
                           Consensus       (401)  AAWS PGLG DT MPPVYSISQ                        VWRSD

                                                  451                                               500
Human DCRS9 SEQ ID NO:12                   (428)  VQFAWKHLLCPDVSYRHLGLLILALLALLTLLGVVLALTCRRPQSGPGPA
     SEQ ID NO:303 from US 6,150,502       (398)  VHFAWKHVLCPDDAPYPTQLLLRSLG---------------S--GR--T
                           Consensus       (451)  V FAWKHLLCPD A       LLI AL                  G

                                                  501                                               550
Human DCRS9 SEQ ID NO:12                   (478)  RPVLLLHAADSEAQRRLVGALAELLRAALGGGRDVIVDLWEGRHVARVGP
     SEQ ID NO:303 from US 6,150,502       (428)  RPVLLLHAADSEAQRRLVGALAELLRTALGGGRDVIVDLWEGTHVARIGP
                           Consensus       (501)  RPVLLLHAADSEAQRRLVGALAELLR ALGGGRDVIVDLWEG HVARIGP

                                                  551                                               600
Human DCRS9 SEQ ID NO:12                   (528)  LPWLWAARTRVAREQGTVLLLWSGADLRPVSGPDPRAAPLLALLHAAPRP
     SEQ ID NO:303 from US 6,150,502       (478)  LPWLWAARERVAREQGTVLLLWNCAGPSTACSGDPQAASLRTLLCAAPRP
                           Consensus       (551)  LPWLWAAR RVAREQGTVLLLW  A          DP AA L  LL AAPRP
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                                                  601                                                650
Human DCRS9 SEQ ID NO:12                          (578) LLLLAYFSRLCAKGDIPPPLRALPRYRLLRDLPRLLRALDARPFAEATSW
      SEQ ID NO:303 from US 6,150,502             (528) -LLLAYFSRLCAKGDIPRPLRALPRYRLLRDLPRLLRALDAQPATLASSW
                            Consensus             (601)  LLLAYFSRLCAKGDIP PLRALPRYRLLRDLPRLLRALDA P   ASSW

                                                  651                                        691
Human DCRS9 SEQ ID NO:12                          (628) GRLGARQRRQSRLELCSRLEREAARLADLG----------
      SEQ ID NO:303 from US 6,150,502             (577) SHLGAKRCLKNRLEQCHLLELEAAKDDYQGSTNSPCGFSCL
                            Consensus             (651)   LGAK     RLE C  LE EAAK    G
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Examiner: Dong Jiang
Daniel M. Gorman (sole)	Art Unit: 1646
Application No.: 09/863,818	Conf. No.: 8990
Filed: May 23, 2001	
For: MAMMALIAN DCRS9; RELATED REAGENTS AND METHODS (as amended)	

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on 9-26-03

by: MELANIE LYONS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

I, Daniel M. Gorman, hereby declare that:

1. I received my B.S. degree from the University of Illinois at Urbana-Champaign in 1984. I have extensive experience in the area of immunology, particularly in the discovery and molecular biology of cytokines and cytokine receptors, as described in my attached curriculum vitae.

2. I have read and understood that the present invention relates to DCRS9 (a.k.a. IL-17RE), a cytokine receptor having significant homology to several IL-17 receptor family members.

3. I have read the Examiner's rejections of Claims 21-26 as presented in the latest Office Action, dated March 27, 2003. I understand that the Examiner has maintained

one rejection of these claims based on a lack of a specific, substantial, or credible utility, as described in the Manual of Patent Examining Procedure §§2107-2107.03 (M.P.E.P., 8th ed., August 2001).

4. The present application contains a number of asserted utilities that are substantial, specific, and credible, e.g., "[t]he cytokine receptor-like proteins will . . . result in modulation of . . . innate immunity response. . . ," "[t]he cytokine receptors . . . and antibodies, should be useful in the treatment of . . . innate immunity . . . ," and "[t]hese data [on DCRS9] demonstrate the receptor is expressed in immune cell types and may be regulated by activation conditions." (page 43, lines 25-27; page 65, lines 10-11; page 80, lines 5-6, of specification). The present application also contains expression data demonstrating increased expression of DCRS9 in mouse lung at t = 4 h after helminth (*Ascaris*) challenge relative to expression in a normal lung pool. Note that innate immunity relates to immune response in the earliest states of infection, see, e.g., Roitt, et al. (1993) *Immunology, 3rd ed.*, Mosby, St. Louis, p. 12.

5. Expression data generated after the priority date of the present application confirm the asserted utility noted above. In particular, data generated by Taqman® quantitative PCR confirm the differential expression of DCRS9 in response to challenge with the helmith, *Nippostrongylus* (Table 1; below).

Table 1. Helminth challenge with *Nippostrongylus*.

Tissue tested	Expression of DCRS9 relative to ubiquitin (ubiquitin expression = 1.0)
Mouse lung untreated.	108.2
Mouse lung infected *Nippostrongylus*.	168.2

6. FACS analysis after the priority date of present application establishes that the ligand of DCRS9 (a.k.a. IL-17RE) is IL-71 (a.k.a. IL-17C) (Appendix I).

7. The FACS analysis data demonstrated that cells transfected with the receptor, DCRS9, specifically bind the ligand, IL-71 (Appendix I, left column). Control studies varying the identity of the transfected receptor demonstrated that cells transfected with other receptors of the IL-17R family, i.e., IL-17RA, IL-17RB, IL-17RC, and IL-17RD, do not specifically bind IL-71 (Appendix I; left column). Control studies varying the ligand demonstrated that DCRS9-transfected cells do not bind IL-73 (a.k.a. IL-17D) (Appendix I; right column).

"Flag IL71" and "Flag IL73," respectively, indicate IL-71 and IL-73 containing a FLAG epitope tag. "hIL17RD-mGCSFR" indicates a fusion protein containing the extracellular part of IL-17R, and the cytosolic part of GCSF receptor (GCSFR) (Appendix I).

8. Data generated after the filing date of the present patent application of the present invention demonstrate that expression of IL-71 increases in response to helminth challenge, as determined by Taqman® quantitative PCR (Table 2; below).

Table 2. Expression of IL-71 in response to helminth challenge.

Tissue tested	Expression of IL-71 relative to ubiquitin (ubiquitin expression = 1.0)
Mouse lung untreated.	0.20
Mouse lung infected *Nippostrongylus.*	7.2

9. Based on the foregoing, one of ordinary skill in the art would believe the asserted utility of DCRS9 as at least a marker for innate immunity, as being substantial, specific, and credible.

10. I hereby attest that I do not have any financial interest in the present application, U.S. Patent Application No. 09/863,818, filed May 23, 2001, Attorney Docket DX01170K.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: September 26, 2003

By: David M. Gorman
Daniel M. Gorman

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Attachment: Curriculum vitae for Daniel M. Gorman (7 pages).

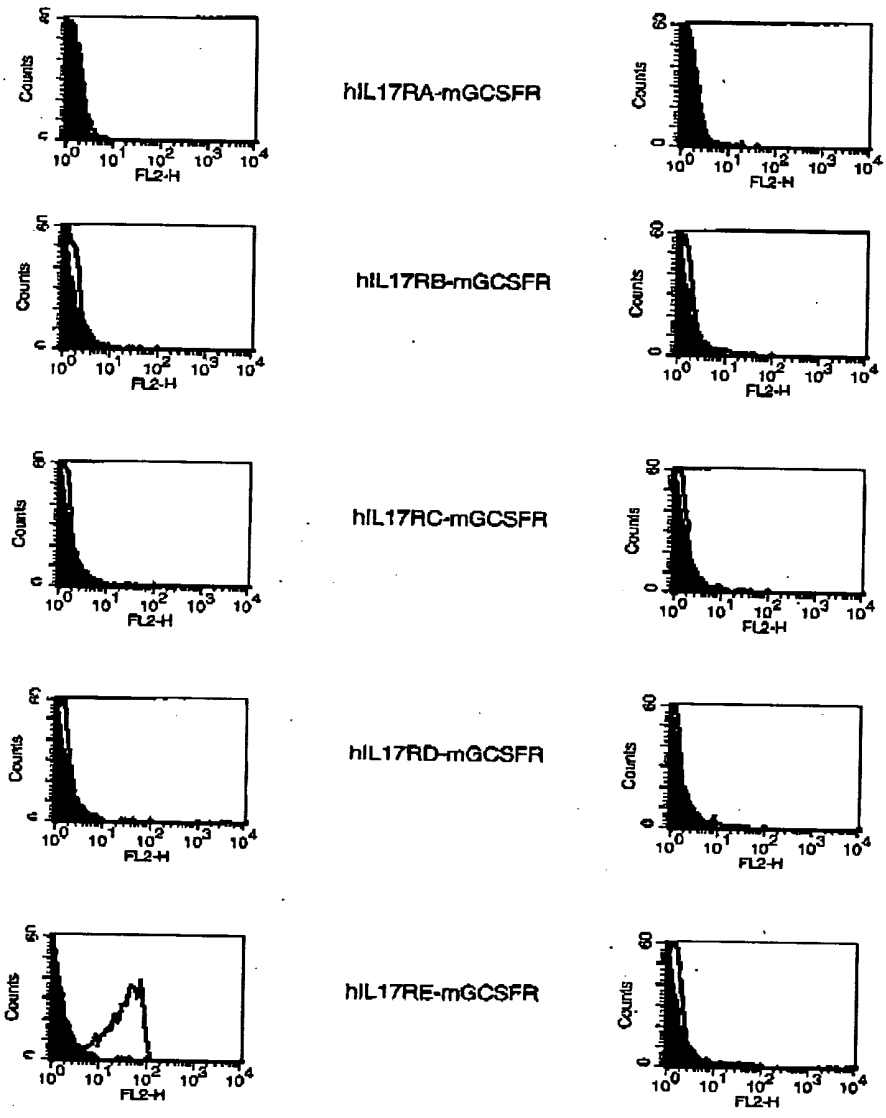
APPENDIX I

LEFT COLUMN

RIGHT COLUMN

Flag IL71

Flag IL73



Curriculum Vitae
Daniel M. Gorman

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email: dan.gorman@dnax.org

Education

B. S. in Microbiology 1984
University of Illinois
Champaign, Urbana, IL.

1983-1984 Senior research project: Phenobarbital induced Cytochrome p450 mRNA induction in rabbit tissues.

Professional Experience

2001-2003. Principal Scientist. DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA. Manager Molecular Biology lab. HGS technology team leader responsible for development of new genes. IL-17 family team member. Protein engineering for structure/function studies. RNAi vector design and validation

2000-2001. Associate Principal Scientist. DNAX Research Institute, Palo Alto, CA. HGS technology team leader responsible for claiming therapeutic proteins. Responsible for directing IL-17 family receptor/ligand matching and investigational research. Developed 14 therapeutic proteins, 48 antibodies, and 16 drug development plans.

1998-2000. Senior Scientist. DNAX Research Institute, Palo Alto, CA. Responsible for discovery of candidate therapeutic proteins using bioinformatics analysis of the public, DNAX, Incyte and HGS DNA sequence databases. Initial characterization of potential therapeutic proteins. Discovery and validation of secreted expression of novel proteins. Managed a group of six research associates.

1995-1998. Research Associate. DNAX Research Institute, Palo Alto, CA. Responsible for DNAX sequencing facility, analysis of newly discovered genes, development of strategies for identifying novel genes. Supervise six staff and summer interns involved in operating DNA automated sequencers, novel cDNA cloning, expression analysis, genomic cloning and characterization. Organize bioinformatics workshop meetings. Implement customized sequence request database and propriatary DNA sequence databases. Responsible for generating gene K.O. constructs.

1988-1995. Scientist III. DNAX Research Institute, Palo Alto, CA. Development of cloning methodologies, purification, sequencing, and cloning of cytokine receptors and molecules involved in signal transduction. Functional characterization of these proteins. Characterization of novel cytokine receptor family including those for human and mouse IL-3, GMCSF, and IL-4. Immuno-affinity purification of proteins associated with IL-2 receptor. Cloning, sequencing and characterization of human and mouse IL-3 receptor and mouse IL-4 receptor.

1986-1987. Scientist II. DNAX Research Institute, Palo Alto, CA. Development of gene transfer techniques and investigation of signal transduction in T-cells. Establishment of a system (Micro-prep, Micro-transfection) for high throughput expression cloning of novel genes.

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2. M34397, Mouse IL-3, GM-CSF, IL-5 common beta receptor mRNA
3. M29854, Mouse IL-4 alpha receptor mRNA
4. M38275, Human IL-3, GM-CSF, IL-5 common beta receptor mRNA
5. M93722, M95501-M95513- Mouse IL-3 specific beta receptor gene
6. M93429, M94136-M94148-Mouse IL-3, GM-CSF, IL-5 common beta receptor gene
7. AF037261, Homo sapiens SH3-containing adaptor molecule-1 mRNA
8. U91746, Homo sapiens IL-10-inducible chemokine (HCC-4) mRNA
9. U87948, Mus musculus hematopoietic neural membrane protein HNMP-1 mRNA
10. U87947, Human hematopoietic neural membrane protein (HNMP-1) mRNA
11. U56145, Human thymic shared antigen-1/stem cell antigen-2 (TSA-1/SCA-2) mRNA
12. AF031826, Mus musculus leukocystatin gene, complete cds
13. AF031825, Mus musculus leukocystatin mRNA, complete cds
14. AF031824, Homo sapiens leukocystatin mRNA, complete cds
15. AJ400844, Mus musculus mRNA for immunoglobulin-like cell surface receptor FDF03
16. AJ400841, Homo sapiens mRNA for immunoglobulin-like cell surface receptor FDF03

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CELLULAR AND MOLECULAR IMMUNOLOGY

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Table 1–1 Effectiveness of Vaccination for Some Common Infectious Diseases

Disease	Maximum Number of Cases	Year of Maximum Number of Cases	Number of Cases in 1992	Percent Change
Diphtheria				
Measles				
Mumps				
Pertussis				
Polio (paralytic)				
Rubella				
Tetanus				
Haemophilus influenzae type b (Hib)				
Hepatitis B				

and biochemical terms. In this chapter, we outline the general features of immune responses and introduce the concepts that form the cornerstones of modern immunology and that recur throughout this book.

INNATE AND ADAPTIVE IMMUNITY

Defense against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity (Fig. 1–1 and Table 1–2). **Innate immunity** (also called natural or native immunity) consists of mechanisms that exist before infection, are capable of rapid responses to microbes, and react in essentially the same way to repeated infections. The principal components of innate immunity are (1) physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; (2) phagocytic cells (neutrophils, macrophages) and natural killer (NK) cells; (3) blood proteins, including members of the complement system and other mediators of inflammation; and (4) proteins called cytokines that regulate and coordinate many of the activities of the cells of innate immunity. The mechanisms of innate immunity are stimulated by structures that are common to groups of related microbes and may not distinguish fine differences between foreign substances. Innate immunity provides the early lines of defense against microbes. The pathogenicity of microbes is, in part, related to their ability to resist the mechanisms of innate immunity.

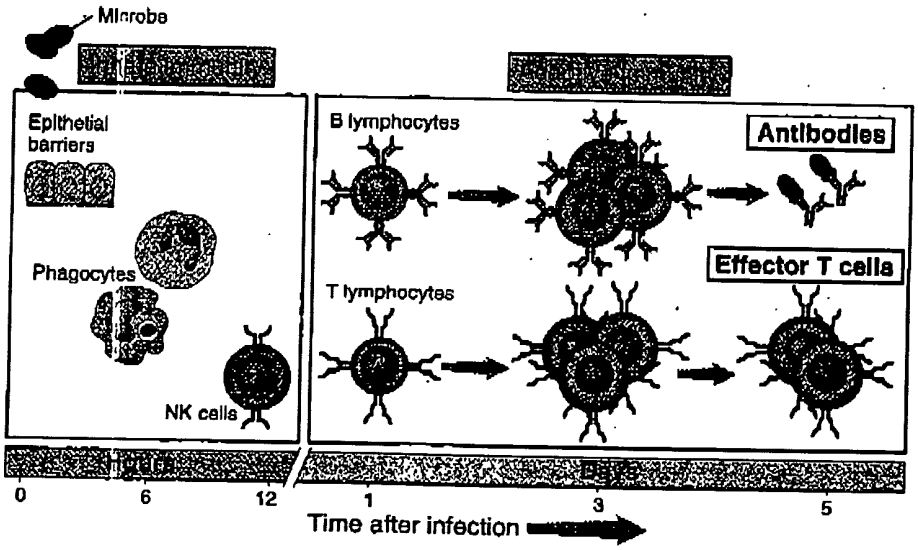
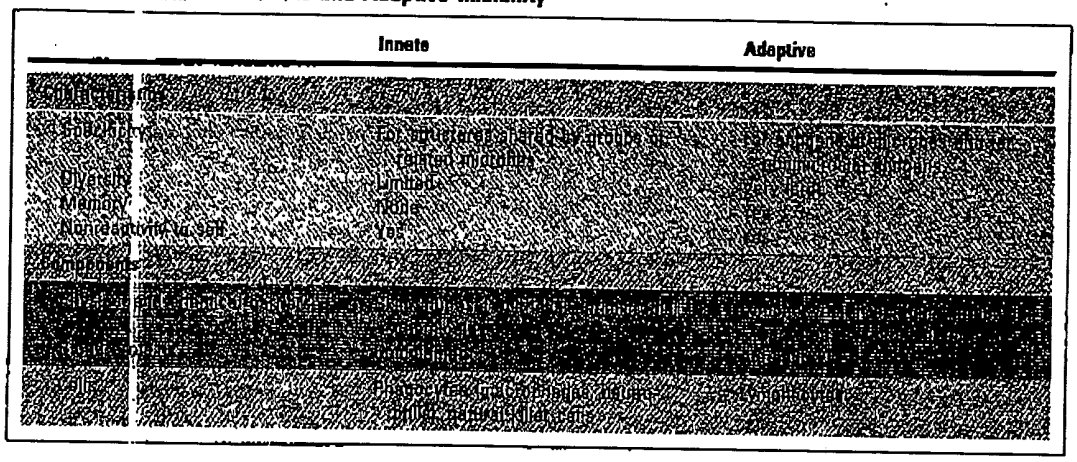


Figure 1–1 Innate and adaptive immunity.
The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and consist of activation of lymphocytes. Only selected mechanisms are shown; for example, the complement system, an important component of innate immunity, is not included. The kinetics of the innate and adaptive immune responses are approximations, and may vary in different infections. NK, natural killer.

Table 1–2 Features of Innate and Adaptive Immunity



In contrast to innate immunity, more highly evolved defense mechanisms are stimulated by exposure to infectious agents and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe. Because this form of immunity develops as a response to infection and adapts to the infection, it is called **adaptive immunity.** The defining characteristics of adaptive immunity are exquisite specificity for distinct macromolecules and an ability to "remember" and respond more vigorously to repeated exposures to the same microbe. Because of its extraordinary capacity to distinguish among different, even closely related, microbes and macromolecules, adaptive immunity is also called **specific immunity.** It is also sometimes called acquired immunity, to emphasize that potent protective responses are "acquired" by experience. The components of adaptive immunity are **lymphocytes** and their products. Foreign substances that induce specific immune responses or are the targets of such responses are called **antigens.**

Innate and adaptive immune responses are components of an integrated system of host defense in which numerous cells and molecules function cooperatively. Two important links exist between innate immunity and adaptive immunity. First, the innate immune response to microbes stimulates adaptive immune responses and influences the nature of the adaptive responses. Second, adaptive immune responses use many of the effector mechanisms of innate immunity to eliminate microbes, and they often function by enhancing the antimicrobial activities of the defense mechanisms of innate immunity. We will return to a more detailed discussion of the mechanisms and physiologic functions of innate immunity in Chapter 12.

The concept that adaptive immune responses enhance and "improve" innate immunity is also reflected in the phylogeny of defense mechanisms (Box 1–1). In invertebrates, host defense against foreign invaders is mediated largely by the mechanisms of innate immunity, including phagocytes and circulating molecules that resemble the plasma proteins of innate immunity in vertebrates. Adaptive immunity, consisting of lymphocytes and antibodies, first appeared in jawed vertebrates and became increasingly specialized with further evolution.

TYPES OF ADAPTIVE IMMUNE RESPONSES

There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity, which are mediated by different components of the immune system and function to eliminate different types of microbes (Fig. 1–2). **Humoral immunity** is mediated by molecules in the blood, called **antibodies,** that are produced by cells called **B lymphocytes.** Antibodies specifically recognize microbial antigens, neutralize the infectivity of the microbes, and target microbes for elimination by various effector mechanisms. Humoral immunity is the principal defense mechanism against extracellular microbes and their toxins because secreted antibodies can bind to these microbes and toxins and assist in their elimination. Antibodies themselves are specialized, and different types of antibodies may activate different effector mechanisms. For example, some types of antibodies promote phagocytosis, and others trigger the release of inflammatory mediators from leukocytes such as mast cells. **Cell-mediated immunity,** also called cellular immunity, is mediated by cells called **T lymphocytes.** Intracellular microbes, such as viruses and some bacteria, survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Defense against such infections is a function of cell-mediated immunity, which promotes the destruction of microbes residing in phagocytes or the lysis of infected cells.

Protective immunity against a microbe may be in-

IMMUNOLOGY

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The cover picture shows a diagrammatic representation of the pentameric polypeptide structure of human IgM (see page 4.5).

chapter 1

Introduction to the Immune System

Our environment contains a great variety of infectious microbes – viruses, bacteria, fungi, protozoa and multicellular parasites. These can cause disease, and if they multiply unchecked they will eventually kill their host. Most infections in normal individuals are short-lived and leave little permanent damage. This is due to the immune system, which combats infectious agents.

Since microorganisms come in many different forms, a wide variety of immune responses are required to deal with each type of infection. In the first instance, the exterior defences of the body present an effective barrier to most organisms, and very few infectious agents can penetrate intact skin (Fig. 1.1). However, many gain access across the epithelia of the gastrointestinal or urogenital tracts. Others can infect the nasopharynx and lung. A small number, such as malaria and hepatitis B, can only infect the body if they enter the blood directly.

The site of the infection, and the type of pathogen, largely determine which immune responses will be effective. The most important distinction is between those which invade the host's cells and those which do not. All viruses, some bacteria and some protozoan parasites replicate inside host cells, and to clear an infection, the immune system must recognize and destroy these infected cells. Many bacteria and larger parasites live in tissues, body fluids or other extracellular spaces, and the responses to these pathogens are quite different. During the course of an infection, however, viruses and other intracellular pathogens must reach their target cells by moving through the blood and tissue fluid. At this time they are susceptible to elements of the immune system which normally counter extracellular pathogens (see Fig. 1.2).

This chapter introduces the basic elements of the immune system and of immune responses, which are detailed in Chapters 2–16. There are various ways in which the immune system can fail, leading to immunopathological reactions, and these are outlined in the second half of the book. However, it is important to stress that the primary function of the immune system is to eliminate infectious agents and to minimize the damage they cause.

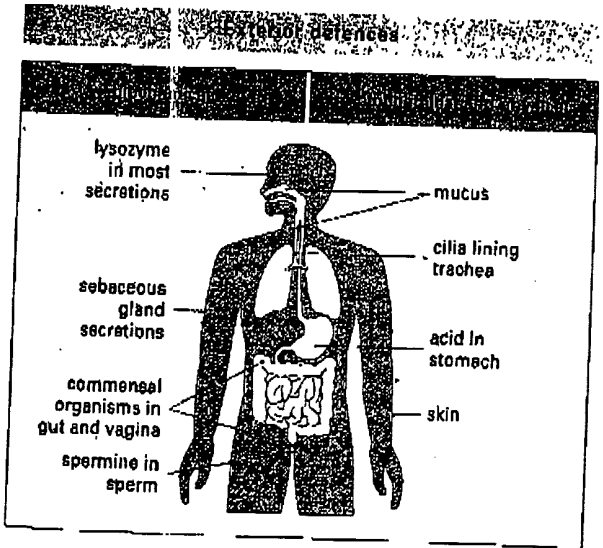


Fig. 1.1 Most of the infectious agents which an individual encounters do not penetrate the body surface, but are prevented from entering by a variety of biochemical and physical barriers. The body tolerates a number of commensal organisms which compete effectively with many potential pathogens.

ADAPTIVE AND INNATE IMMUNITY

Any immune response involves, firstly, recognition of the pathogen or other foreign material, and secondly, mounting a reaction against it, to eliminate it. Broadly speaking, the different types of immune response fall into two categories: innate (or non-adaptive) responses, and adaptive immune responses. The important difference between these is that an adaptive immune response is highly specific for a particular pathogen. Moreover, the response improves with each successive encounter with the same pathogen: in effect the adaptive immune system 'remembers' the infectious agent and can prevent it from causing disease later. For example, diseases such as measles and diphtheria induce adaptive immune responses which generate a lifelong immunity following an infection. The two key features of the adaptive immune response are thus specificity and memory.

Immune responses are produced primarily by leucocytes, of which there are several different types. One important group of leucocytes are the phagocytic cells, such as the monocytes, macrophages and polymorphonuclear neutrophils. They bind to microorganisms, internalize them and destroy them. Since they use primitive non-specific recognition systems, which allow them to bind to a variety of microbial products, they are mediating innate immune responses. In effect they act as a first line of defence against infection.

Another important set of leucocytes are the lymphocytes. These cells are central to all adaptive immune responses, since they specifically recognize individual pathogens, whether they are inside host cells or outside in the tissue fluids or blood. In fact there are several different types of lymphocyte, but they fall into two basic categories – T lymphocytes (or T cells) and B lymphocytes (or B cells). B cells combat extracellular pathogens and their products by releasing antibody, a molecule which specifically recognizes and binds to a particular target molecule, called the antigen. The antigen may be a molecule on the surface of a pathogen, or a toxin which it produces. T lymphocytes have a wider range of activities. Some are involved in the control of B lymphocyte development and antibody production. Another group of T cells interacts with phagocytic cells to help them destroy pathogens they have taken up. A third set of T lymphocytes recognizes cells infected by virus and destroys them.

In practice there is a considerable amount of interaction between the lymphocytes and phagocytes. For example, some phagocytes can take up antigens and show them to T lymphocytes in a form they can recognize, a process which is called antigen presentation. In turn the T lymphocytes release soluble factors (cytokines), which activate the phagocytes and cause them to destroy the pathogens they have internalized. In another interaction phagocytes use antibodies released by B lymphocytes to allow them to recognize pathogens more effectively (Fig. 1.3). One consequence of these interactions, is that most immune responses to infectious organisms are made up of a variety of innate and adaptive components. In the earliest stages of infection, innate responses predominate, but later the lymphocytes start to generate adaptive immune responses. They then remember the pathogen, and mount more effective and rapid responses should the individual become reinfected with that agent.

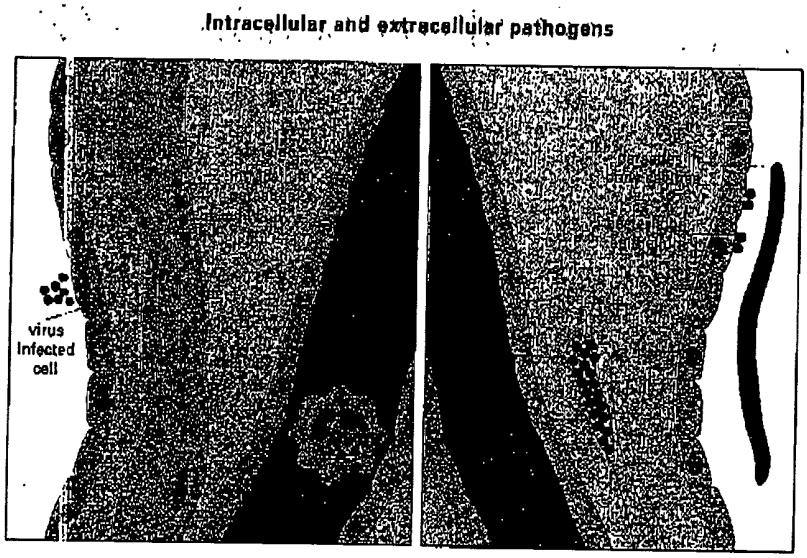
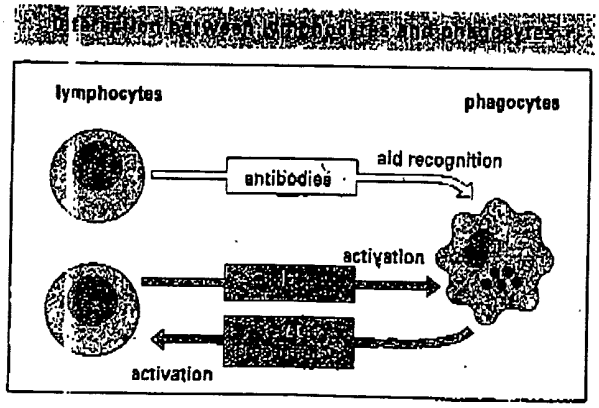


Fig. 1.2 The immune system must recognize and react against pathogens in a number of different locations. For example, viruses must invade cells to reproduce, while [illegible]



Selectin Blockade Prevents Antigen-induced Late Bronchial Responses and Airway Hyperresponsiveness in Allergic Sheep

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Antigen challenge can elicit an allergic inflammatory response in the airways that involves eosinophils, basophils, and neutrophils and that is expressed physiologically as a late airway response (LAR) and airway hyperresponsiveness (AHR). Although previous studies have suggested that E-selectin participates in these allergic airway responses, there is little information concerning the role of L-selectin. To address this question, we examined the effects of administering an L-selectin-specific monoclonal antibody, DU1-29, as well as three small molecule selectin binding inhibitors, on the development of early airway responses (EAR), LAR and AHR in allergic sheep undergoing airway challenge with *Ascaris suum* antigen. Sheep treated with aerosol DU1-29 before antigen challenge had a significantly reduced LAR and did not develop postchallenge AHR. No protective effect was seen when sheep were treated with a nonspecific control monoclonal antibody. Treatment with DU1-29 also reduced the severity of the EAR to antigen. Similar results were obtained with each of the three small molecule selectin inhibitors at doses that depended on their L-, but not necessarily E-selectin inhibitory capacity. The inhibition of the EAR with one of the inhibitors, TBC-1269, was associated with a reduction in histamine release. Likewise, treatment with TBC-1269 reduced the number of neutrophils recovered in bronchoalveolar lavage (BAL) during the time of LAR and AHR. TBC-1269, given 90 min after antigen challenge also blocked the LAR and the AHR, but this protection was lost if the treatment was withheld until 4 h after challenge, a result consistent with the proposed time course of L-selectin involvement in leukocyte trafficking. These are the first data indicating that L-selectin may have a unique cellular function that modulates allergen-induced pulmonary responses. **Abraham WM, Ahmed A, Sabater JR, Lauredo IT, Botvinnikova Y, Bjercke RJ, Hu X, Revelle BM, Kogan TP, Scott IL, Dixon RAF, Yeh ETH, Beck PJ. Selectin blockade prevents antigen-induced late bronchial responses and airway hyperresponsiveness in allergic sheep.**

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Airway inflammation is thought to contribute to the chronic airway hyperresponsiveness of asthma. In the laboratory, the development of late bronchial responses following inhalation challenge with specific antigen may be the initial physiologic sign of this inflammatory response. Late airway responses (LAR) are often followed by a prolonged period of airway hyperresponsiveness (AHR), which reflects a continued inflammatory process initiated by this single challenge (1). The precise molecular events that initiate and amplify this antigen-induced inflammatory response have not been entirely defined, but the accumulation and activation of leukocytes in response to inflammatory stimuli are part of a complex cascade of events that is dependent upon the binding of leukocyte and endothelial cell surface adhesion receptors. These adhesion receptors include the selectins, several members of the IgG super family (vascular cell adhesion molecule [VCAM] and intercellular adhesion molecule [ICAM]), together with the β1 and β2 integrins (membrane attack complex [Mac-1], lymphocyte-function-associated antigen-1 [LFA-1], and very late antigen-4 [VLA-4]). Thus, E-selectin (endothelial-leukocyte adhesion molecule-1 [ELAM-1]), VLA-4, VCAM-1, and ICAM-1 have all been implicated in the inflammatory responses that follow allergen challenge, because treatment of experimental animals with monoclonal antibodies or small molecule inhibitors against these adhesion proteins can block either the LAR, the postchallenge AHR, or both (2–7).

E-, L-, and P-selectin are adhesion proteins that are important in the initial processes modulating the trafficking of leu-

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TABLE 1

SUMMARY OF TREATMENT RESULTS*

Treatment and Mode of Administration	n	Time of Treatment	EAR (%) + Drug	EAR (%) + Vehicle	LAR (%) + Drug	LAR (%) + Vehicle	PC Ratio + Drug	PC Ratio + Vehicle
30 mg TBC-265 (aerosol)	2	30 min prior to challenge	56 ± 21†	173 ± 39	16 ± 6†	108 ± 14	0.45	0.31
15 mg TBC-265 (aerosol)	2	30 min prior to challenge	183 ± 32†	199 ± 37	150 ± 23‡	112 ± 27	0.44	0.60
10 mg TBC-1269 (aerosol)	5	30 min prior to challenge	91 ± 22†	199 ± 29	36 ± 4†	145 ± 4	0.89	0.45
4 mg TBC-1269 (aerosol)	3	30 min prior to challenge	97 ± 24†	247 ± 49	85 ± 9†	135 ± 21	0.54	0.51
40 mg TBC-1269 (aerosol)	2	2 h prior to challenge	148 ± 12†	416 ± 110	46 ± 15†	214 ± 83	1.04	0.71
3 mg/kg TBC-1269 (IV)	4	15 min prior to challenge	100 ± 13†	172 ± 29	18 ± 6†	69 ± 18	0.97	0.50
10 mg TBC-1269 (aerosol)	5	90 min after challenge	175 ± 28†	222 ± 41	18 ± 6†	201 ± 4	0.89	0.48
10 mg TBC-1269 (aerosol)	4	4 h after challenge	179 ± 20†	233 ± 33	88 ± 8†	169 ± 38	0.43	0.45
5 mg MBPA (aerosol)	2	30 min prior to challenge	91 ± 25†	189 ± 58	12 ± 6†	156 ±15	1.10	0.52
2.5 mg MBPA (aerosol)	2	30 min prior to challenge	123 ± 28†	187 ± 44	23 ± 4†	130 ± 13	1.01	0.42
1 mg MBPA (aerosol)	2	30 min prior to challenge	127 ± 29†	195 ± 47	81 ± 7†	105 ± 14	0.60	0.43
10 mg DU1-29 (aerosol)	4	30 min prior to challenge	71 ± 13†	184 ± 20	14 ± 4†	112 ± 14	1.02	0.43
10 mg MD6 (aerosol)	4	30 min prior to challenge	146 ± 14†	212 ± 33	118 ± 11†	118 ± 24	0.52	0.50

Definition of abbreviations: EAR = early airway response; LAR = late airway response; expressed as percent change from baseline; PC = the ratio of post/prechallenge value of PC_{400}. A ratio of 1 indicates that there was no change in the airway responsiveness, whereas a ratio below 1 indicates the development of airway hyperresponsiveness.

* Values are mean ± standard error.

† $p < 0.05$ and ‡ $p > 0.1$ as determined by analysis using a two-tailed, unpaired, heteroscedastic Student's *t* test with each indicated group compared with results obtained after antigen challenge of the same animals without drug treatment.

kocytes to areas of vascular trauma or inflammation. P-selectin (CD62P) is expressed upon the surface of activated platelets and endothelial cells, E-selectin (CD62E) upon the surface of activated endothelial cells, and *L*-selectin (CD62L) is expressed upon the surface of eosinophils, neutrophils, monocytes, and lymphocytes. The initial step in leukocyte migration from blood into extravascular tissue is thought to be adherence to the surface of endothelial cells that line vessel walls. This initial adherence is thought to be dependent on selectin-mediated processes (8–10). Based on these data, we hypothesized that treatment with selectin inhibitors should prevent the subsequent inflammatory events, i.e., the LAR and the AHR associated with allergen challenge. Partial support for this hypothesis comes from previous studies in primates that indicate that pretreatment with a monoclonal antibody to E-selectin blocks LAR (2). However, these studies did not assess the effect of E-selectin blockade on the post-antigen-induced AHR nor did they evaluate the effect of giving such drugs after antigen challenge. This timing may be critically important because selectins are involved early on in the inflammatory cascade. Thus, one would speculate that selectin inhibitors need to be given either before antigen challenge or soon after challenge (1–2 h) to show their maximal effect.

To test this hypothesis, confirm mechanism of action, and extend previous findings, we have utilized an anti-L-selectin blocking antibody and several small molecule selectin inhibitors and evaluated the effect of these compounds in the sheep model of allergic bronchoconstriction (11). In this model, airway challenge with specific antigen results in an early airway response (EAR), a LAR, and AHR for up to 2 wk after challenge (1, 11). Our results indicate that the anti-L-selectin antibody and small molecule selectin binding inhibitors are able to block the LAR and AHR that follow antigen challenge in this animal model. These changes are associated with a reduction in the cellular inflammatory response as estimated by bronchoalveolar lavage (BAL).

METHODS

Materials

Tissue culture media, dialyzed fetal calf serum (FCS), phosphate-buffered saline (PBS), and antibiotics were obtained from Life Sciences Inc. (Gaithersburg, MD) and FCS from Hyclone (Logan, UT). The anti-L-selectin antibody (SK11) was purchased from Becton-Dickinson (San Jose, CA), and anti-L-selectin antibody (DREC56) and the biotinylated conjugate were purchased from Endogen (Cambridge, MA). The DU1-29 hybridoma was obtained from the American Type Culture Collection (Rockville, MD), and antibody used in experiments was purified from ascites as described subsequently. The anti-P-selectin antibody (AC1.2) was purchased from Becton-Dickinson. Unless specifically stated, other immunochemicals were purchased

TABLE 2

SELECTIN INHIBITORS*

Compound Name	IC_{50} Human E-Selectin	IC_{50} Human P-Selectin	IC_{50} Human L-Selectin	E:L Inhibitory Capacity	M_r	Description and/or Reference
sLe^a	170 μM	160 μM	193 μM	0.88	821	(22)
TBC-265	150 μM	173 μM	177 μM	0.85	390	(23)
TBC-1269	105 μM	17 μM	87 μM	1.21	906	(12)
MBPA	382 μM	489 μM	115 μM	3.32	208	(24)
DU1-29	No effect @ 50 μg/ml	No effect @ 50 μg/ml	0.3 μg/ml – 2 nM		~150,000	Anti-sheep L-selectin antibody (25, 26)
MD6	ND	ND	No effect @ 50 μg/ml		~150,000	Nonspecific negative control antibody

* Selectin IgG fusion protein were assayed for adherence to sLe^a glycolipids prepared as described (27). Fusion protein binding in the absence of compound is defined as 100% and that for the mock control wells is designated 0%. The data represent the mean of two independent experiments run in duplicate. The inhibitory capacity was determined from respective IC_{50} values against human selectins. The higher the ratio, the more potent the compound is against L-selectin. Assays were performed as previously described (28).

from Calbiochem (San Diego, CA). Flexible 96-well assay plates and Probind 96-well ELISA plates were purchased from Falcon (Becton-Dickinson).

Chemicals

TBC-265 and TBC-1269 were synthesized as described elsewhere (12, 13). MBPA ([3-(4-methoxybenzoyl) propionic acid]) was purchased from Aldrich (Milwaukee, WI). Stock solutions used for animal experiments were freshly prepared in sterile, pyrogen-free 0.9% NaCl (VWR Scientific Products, West Chester, PA) and when necessary adjusted to pH 7.3 with NaOH.

Antibody Purification

Mice were primed by intraperitoneal injection of 0.1 ml pristane 7 to 10 d prior to intraperitoneal injection of 10^7 hybridoma cells expressing DU1-29 antibody or MD6 antibody. Ten to 14 d after injection, peritoneal ascites fluid was evacuated by aspiration. After brief centrifugation at 600 × *g*, ascites was frozen and stored at −80° C until mouse IgG was fractionated from ascites by passing the diluted fluid (1:2 into PBS) over a 10-ml protein G (Amersham Pharmacia, Biotech, Uppsala, Sweden) column and eluting as recommended by the manufacturer. Eluent was extensively dialyzed against 10 mM ammonium acetate (pH 7.5) and lyophilized. The amount of recovered antibody was determined by ELISA using the procedures of Harlow and Lane (14).

Animal Preparation

Sheep weighing between 27 and 36 kg that had previously been shown to develop both early and late bronchial responses to inhaled *Ascaris suum* antigen were conscious and were restrained in a modified shopping cart in the prone position with their heads immobilized as previously described (4, 11). After topical anesthesia of the nasal passages with 2% lidocaine, a balloon catheter was advanced through one nostril into the lower esophagus. The animals were intubated with a cuffed endotracheal tube through the other nostril with a flexible fiberoptic bronchoscope as a guide. All protocols used in this study

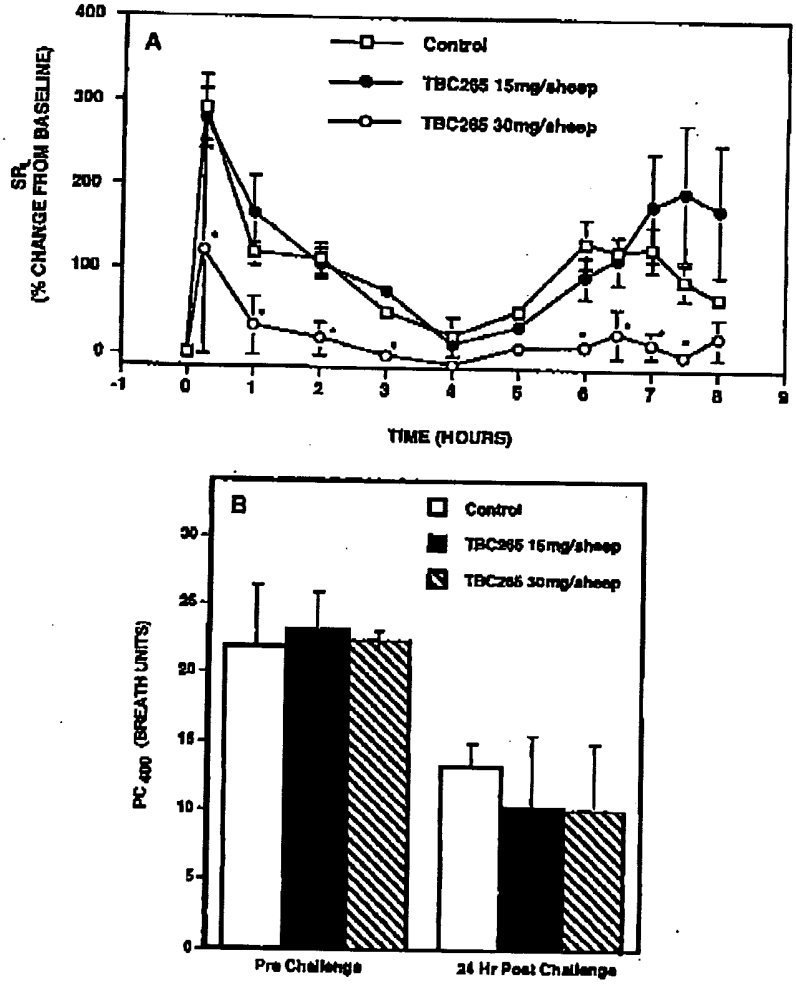

Figure 1. Effect of aerosol treatment with TBC-265 on antigen-induced airway responses. Values are mean ± SE for two sheep. *$p < 0.05$ versus control. (*A*) Effect of antigen challenge on the changes in SR_L in sheep with and without treatment with TBC-265. TBC-265 was given as an aerosol 30 min before challenge. TBC-265 (30 mg) gave significant protection against the antigen-induced early and late increases in SR_L. (*B*) Effect of TBC-265 on antigen-induced airway hyperresponsiveness in allergic sheep. Neither the high- nor low-dose treatment blocked the post-antigen-induced airway hyperresponsiveness (i.e., the fall in PC_{400}).

were approved by the Mount Sinai Medical Center Animal Research Committee, which is responsible for assuring the humane care and use of experimental animals.

Measurement of Airway Mechanics

Breath by breath determination of mean pulmonary flow resistance (R_L) was measured with the esophageal balloon technique that has been described previously by us (4, 7). The mean of at least five breaths, free of swallowing artifact, was used to obtain R_L in cm H_2O/L/s. Immediately after the measurement of R_L, thoracic gas volume (Vtg) was measured in a constant-volume body plethysmograph to obtain specific lung resistance ($SR_L = R_L \times Vtg$) in liter × cm H_2O/L/s (4, 7).

Aerosol Delivery System

Aerosols were generated using a disposable medical nebulizer that provided an aerosol with a mass median aerodynamic diameter of 3.2 μm as determined by a cascade impactor. The nebulizer was connected to a dosimeter system, consisting of a solenoid valve and a source of compressed air (20 psi). The output of the nebulizer was directed into a plastic T-piece, one end of which was connected to the inspiratory port of a respirator. The solenoid valve was activated for 1 s at the beginning of the inspiratory cycle of the respirator. Aerosols were delivered at a tidal volume of 500 ml and a rate of 20 breaths per minute (4, 7).

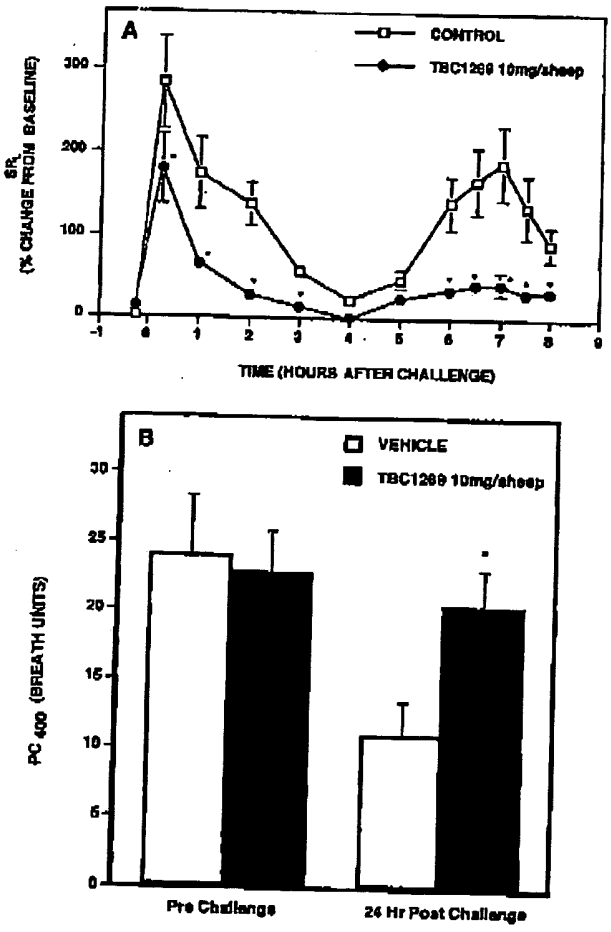

Figure 2. Effect of aerosol treatment with TBC-1269 on antigen-induced airway responses. Values are mean ± SE for five sheep. *$p < 0.05$ versus control. (*A*) Effect of antigen challenge on the changes in SR_L in sheep with and without treatment with TBC-1269. TBC-1269 (10 mg) was given as an aerosol 30 min before challenge. The compound significantly reduced the early and blocked the late antigen-induced increases in SR_L. (*B*) Effect of aerosol treatment with TBC-1269 on antigen-induced airway hyperresponsiveness in allergic sheep. A single 10-mg treatment with TBC-1269 before antigen challenge blocked the post-antigen-induced airway hyperresponsiveness (i.e., the fall in PC_{400}) 24 h later.

Airway Responsiveness

Airway responsiveness was determined from cumulative concentration–response curves to inhaled carbachol as previously described (4, 7). SR_L was measured immediately after inhalation of buffer and after each consecutive administration of 10 breaths of increasing concentrations of carbachol (0.25, 0.5, 1.0, 2.0, and 4.0% wt/vol PBS). The cumulative carbachol concentration (in breath units [BU]) that increased SR_L by 400% over the postsaline value (PC_{400}) was calculated from the dose–response curve. One BU was defined as one breath of a 1% wt/vol carbachol aerosol solution (4, 7).

BAL

The distal tip of a fiberoptic bronchoscope was wedged into three randomly selected subsegmental bronchi. Lung lavage was performed by infusion and aspiration of 30-ml aliquots of PBS (Sigma; pH 7.4) at 39° C. A different airway was used for each 30-ml aliquot (total 90 ml at each time point). The effluents were combined and strained through gauze to remove mucus. The total number of cells was counted in a hemocytometer from a sample of unconcentrated lavage using phase microscopy. The effluent was then centrifuged at 420 × *g* for 15 min and the cell pellet was resuspended in PBS. A cytocentrifuge separation was made and stained by Wright-Giemsa to identify cell populations. Five hundred cells per slide were enumerated to establish the differential cell count (100×; oil objective). Cell categories included macrophages, lymphocytes, neutrophils, and eosinophils (4).

Protocol (*In Vivo* Studies)

All studies were done in crossover fashion such that each sheep served as its own control. The same general protocol was used for all studies, except that the dosage and time of treatment with the different inhibitors were varied. Details of the doses and routes of administration of the compounds are given in Table 1. This basic protocol consisted of obtaining baseline dose–response curves to aerosol carbachol (i.e., PC_{400}) 1 to 3 d before antigen challenge. Then, on the antigen challenge day, baseline values of SR_L were obtained after which the sheep were challenged with *A. suum* antigen. Measurements of SR_L were obtained immediately after challenge, hourly from 1 to 6 h after challenge and on the half-hour from 6.5–8 h after challenge. Measurements of SR_L were obtained 24 h after challenge followed by the 24-h postchallenge determination of PC_{400}. Drug and vehicle control trials were separated by at least 2 wk.

Anti-Inflammatory Studies

To assess the anti-inflammatory capacity of TBC-1269, six sheep were challenged on two separate occasions, once without (PBS; placebo) and once after pretreatment (−0.5 h) with 10 mg TBC-1269 aerosol in a randomized crossover fashion. In these studies, a baseline BAL was performed before treatment and then 6.5 h and 24 h after antigen challenge. Total cell and cell differential responses were expressed as cells/ml lavage return. In addition to the cell response, we measured tissue kallikrein activity in BAL, which has been previously shown by us to be a marker of inflammation (15). Tissue kallikrein was measured in aliquots (stored at −70° C until analysis) of the cell-free supernatant from each of the BAL samples using a modification of the procedure previously described by us (15). Briefly, tissue kallikrein was determined in unconcentrated samples from BAL using a microtiter assay. A volume of 150 μl BAL was incubated with 25 μl of trypsin (20 μg/ml in tris [hydroxymethyl] aminomethane [Trizma] buffer 0.05 M, pH 8.2) for 15 min at 37° C. Then, 25 μl of soy bean trypsin inhibitor (4 mg/ml in 0.1 M Trizma buffer, pH 8.2) was added followed by 100 μl of substrate DL Val-Leu-Arg p-nitroanilide (pNA) dissolved in Trizma buffer 0.05 M, pH 8.2, with 0.05% albumin and

incubated for 24 h in a humidified CO_2 incubator. The values were reported as the change in optical density between zero and 24 h, measured at a wavelength of 405 nM. All assays were done in duplicate.

Histamine Release

To determine whether TBC-1269 had an effect on mast cell degranulation, six sheep were challenged with antigen on two separate occasions, once without (PBS; placebo) and once after pretreatment (−0.5 h) with 10 mg TBC-1269 aerosol in a randomized crossover fashion. In these studies, a baseline BAL (one 30-ml aliquot) was performed before treatment and then 30 min and 60 min after antigen challenge. The lavage return was centrifuged to remove the cells, and aliquots of the supernatants were analyzed for histamine using a commercially available enzyme immunoassay kit according to the manufacturer's instructions. The sensitivity of the assay is 0.5 nM. Samples were done in duplicate (Immunotech, Marseille, France).

Statistical Analyses

For all airway mechanics studies, all probabilities were determined using two-tailed, unpaired, heteroscedastic Student's *t* tests performed using Microsoft Excel version 5.0a. Nonparametric statistics were used to analyze the BAL cell results. For the cell responses, Friedman's two-way analysis of variance was used to determine overall effects followed by Wilcoxon's test to distinguish differences at individual time points (two-tailed). For the tissue kallikrein measurements, the data were $\log_{10}$ transformed and then analyzed by a two-way analysis of variance to determine overall effects. Differences at individual time points were determined by paired *t* test (two-tailed) (Systat for Windows, Version 5; SYSTAT, Inc., Evanston, IL). The histamine results were also $\log_{10}$ transformed and then analyzed by paired *t* test. Because the functional results suggested that mediator release was inhibited after treatment, we used a one-tailed test to determine significance (16).

RESULTS

The inhibitors discussed in the text together with the *in vitro* efficacies that were determined for inhibition of human selectin binding and the physical descriptions of the compounds or references detailing those descriptions are listed in Table 2. As illustrated, each of three low-molecular-weight inhibitors blocks all selectins, but each has a different ratio of E:L selec-

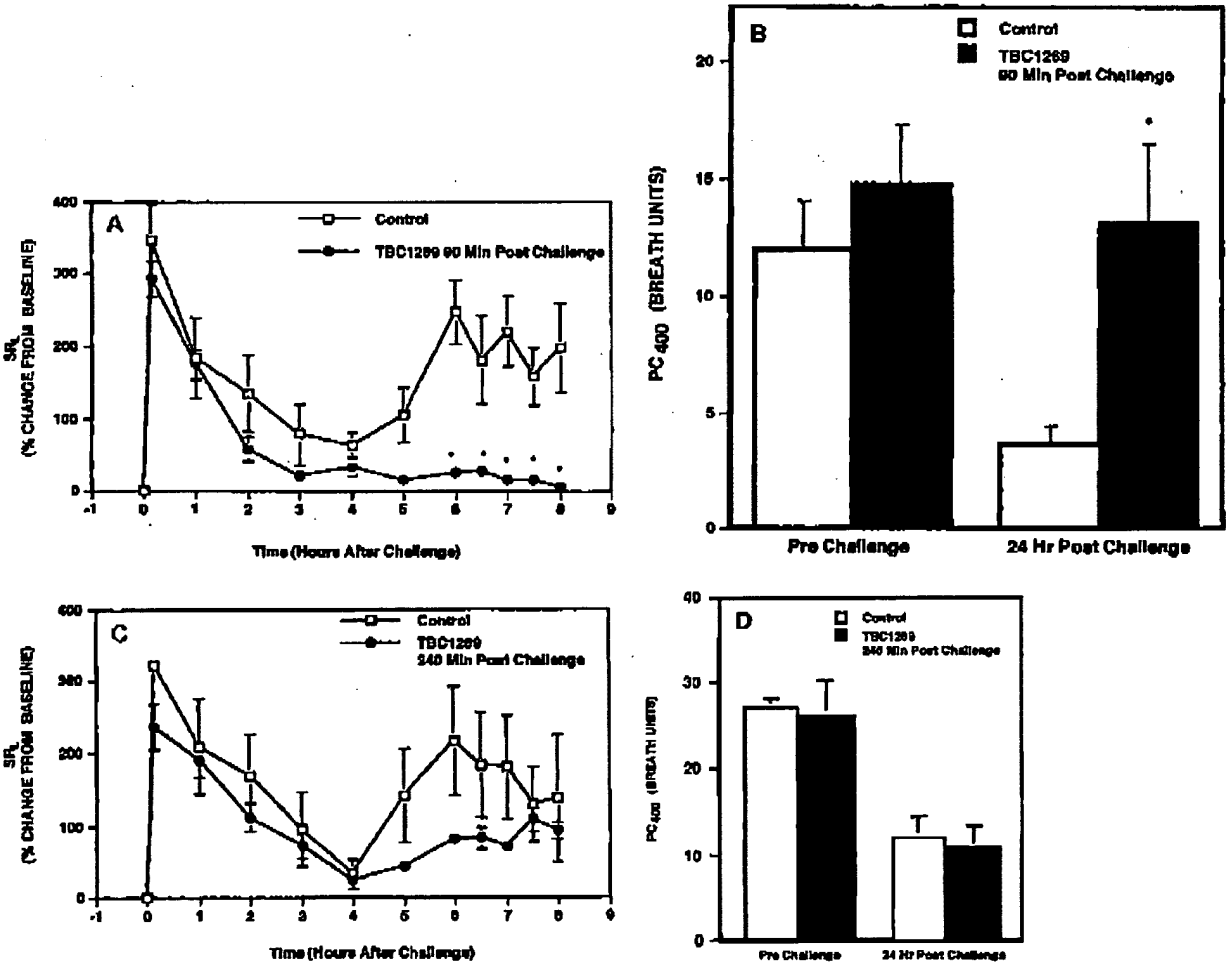

Figure 3. Effect of TBC-1269 when administered after antigen challenge. Values are mean ± SE. $^*p < 0.05$ versus control. (*A*) When TBC-1269 (10 mg) was given 90 min after challenge (n = 5), it provided significant protection against the late increases in SR_L and (*B*) antigen-induced airway hyperresponsiveness. If treatment was withheld until 240 min after antigen challenge, (*C*) this protection against the late increases in SR_L and (*D*) antigen-induced airway hyperresponsiveness was lost (n = 4).

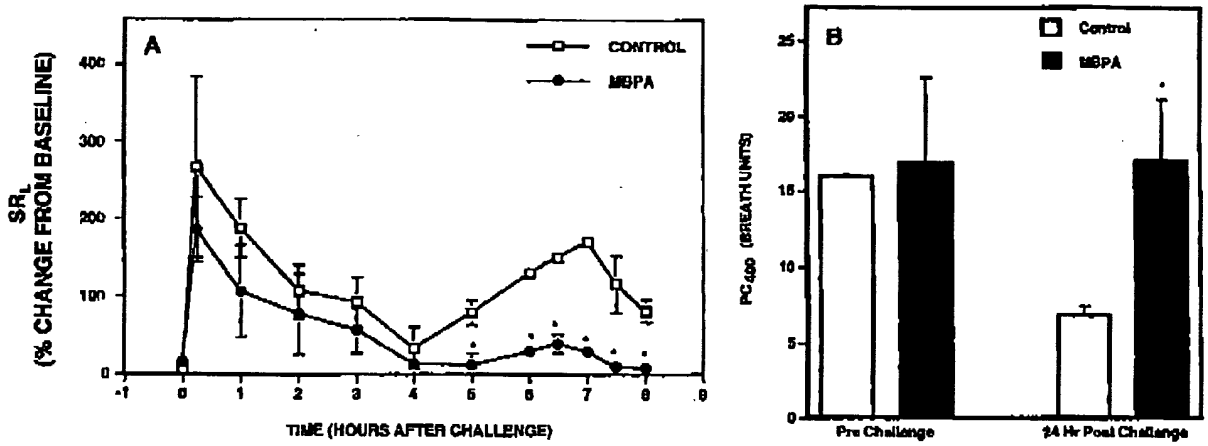

Figure 4. Effect of aerosol treatment with MBPA when administered before antigen challenge. Values are mean ± SE. *$p < 0.05$ versus control. (*A*) Effect of antigen challenge on the changes in SR_L in sheep with and without treatment with MBPA. MBPA (2.5 mg; n = 2) was given as an aerosol 30 min before challenge. The compound significantly reduced the early and blocked the late antigen-induced increases in SR_L. (*B*) Effect of aerosol treatment with MBPA on antigen-induced airway hyperresponsiveness in allergic sheep. A single 2.5-mg treatment with MBPA before antigen challenge blocked the post-antigen-induced airway hyperresponsiveness (i.e., the fall in PC_{400}).

tin inhibitory activity based on the values of the concentration that inhibits binding by 50% (IC_{50}) for the respective selectins. As will be shown, these ratios are predictive of the doses of the different inhibitors necessary to show activity in the *in vivo* experiments, i.e., as the E:L selectin inhibitory ratio increases, the dose of compound required to give protection against the pathophysiological endpoints falls. Also shown are the studies that demonstrate that a commercially available anti-sheep L selectin antibody, DU1-29, is able to recognize and inhibit human L-selectin binding to sheep neutrophils, whereas the control antibody, MD6, which does not recognize L-selectin, does not inhibit binding.

Initial *in vivo* studies indicated that pretreatment with 30 mg, but not 15 mg, aerosolized TBC-265 significantly reduced the EAR and blocked the LAR after antigen challenge (Figure 1A and Table 1). Although the 30-mg dose of TBC-265 was effective in blocking the LAR, there was no subsequent effect on the 24-h AHR as evidenced by the fall in the PC_{400} (Figure 1B).

A similar series of experiments were then conducted with a

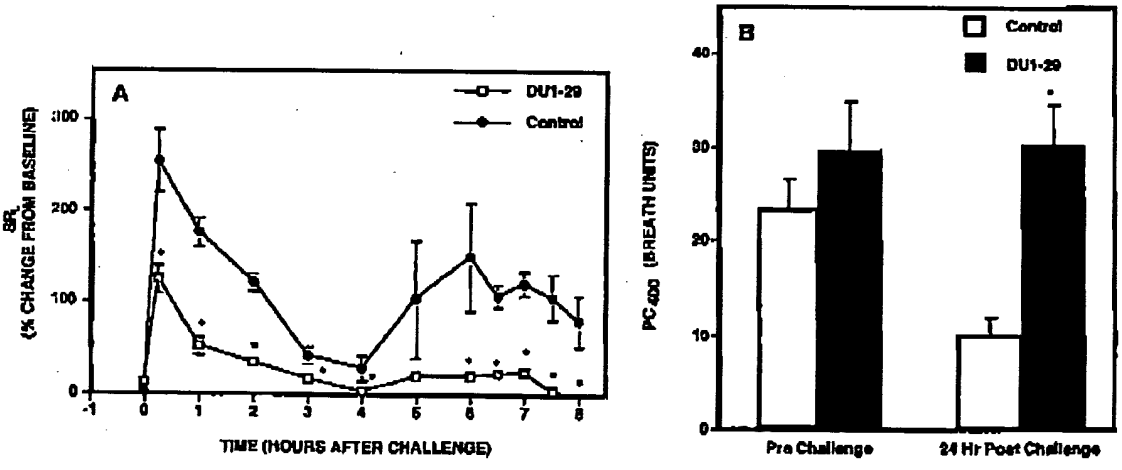

Figure 5. Effect of aerosol treatment with a specific antibody to sheep L-selectin (DU1-29) on antigen-induced changes when administered before antigen challenge. Values are mean ± SE for four sheep. *$p < 0.05$ versus control. (*A*) Effect of antigen challenge on the changes in SR_L in sheep with and without treatment with DU1-29. DU1-29 (10 mg) was given as an aerosol 30 min before challenge. The antibody significantly reduced the early and blocked the late antigen-induced increases in SR_L. (*B*) Effect of aerosol treatment with DU1-29 on antigen-induced airway hyperresponsiveness in allergic sheep. A single 10-mg treatment with DU1-29 before antigen challenge blocked the post-antigen-induced airway hyperresponsiveness (i.e., the fall in PC_{400}).

second, structurally related selectin inhibitor, TBC-1269, that displayed greater *in vitro* ability to inhibit E-, P-, and L-selectin binding. As predicted from the *in vitro* IC_{50} values, a lower dose (10 mg) of TBC-1269 administered as an aerosol to the sheep 30 min before antigen challenge, significantly reduced the EAR and blocked the LAR (Figure 2A). However, in addition to inhibiting the LAR, treatment with TBC-1269 also blocked 24-h AHR ($p < 0.02$) (Figure 2B). The protective effects of TBC-1269 were lost if the dose was reduced (4 mg/sheep, Table 1). We found that the pretreatment time for nebulized TBC-1269 could be extended to 2 h, if the dose was increased appropriately (i.e., four times the 30-min pretreatment dose) and that TBC-1269 was also effective in blocking the three physiologic endpoints, EAR, LAR, and AHR when administered intravenously at 3 mg/kg (Table 1).

Because selectin expression and activation are part of the initial inflammatory event, one would expect that treatment with TBC-1269 should be effective if given after antigen chal-

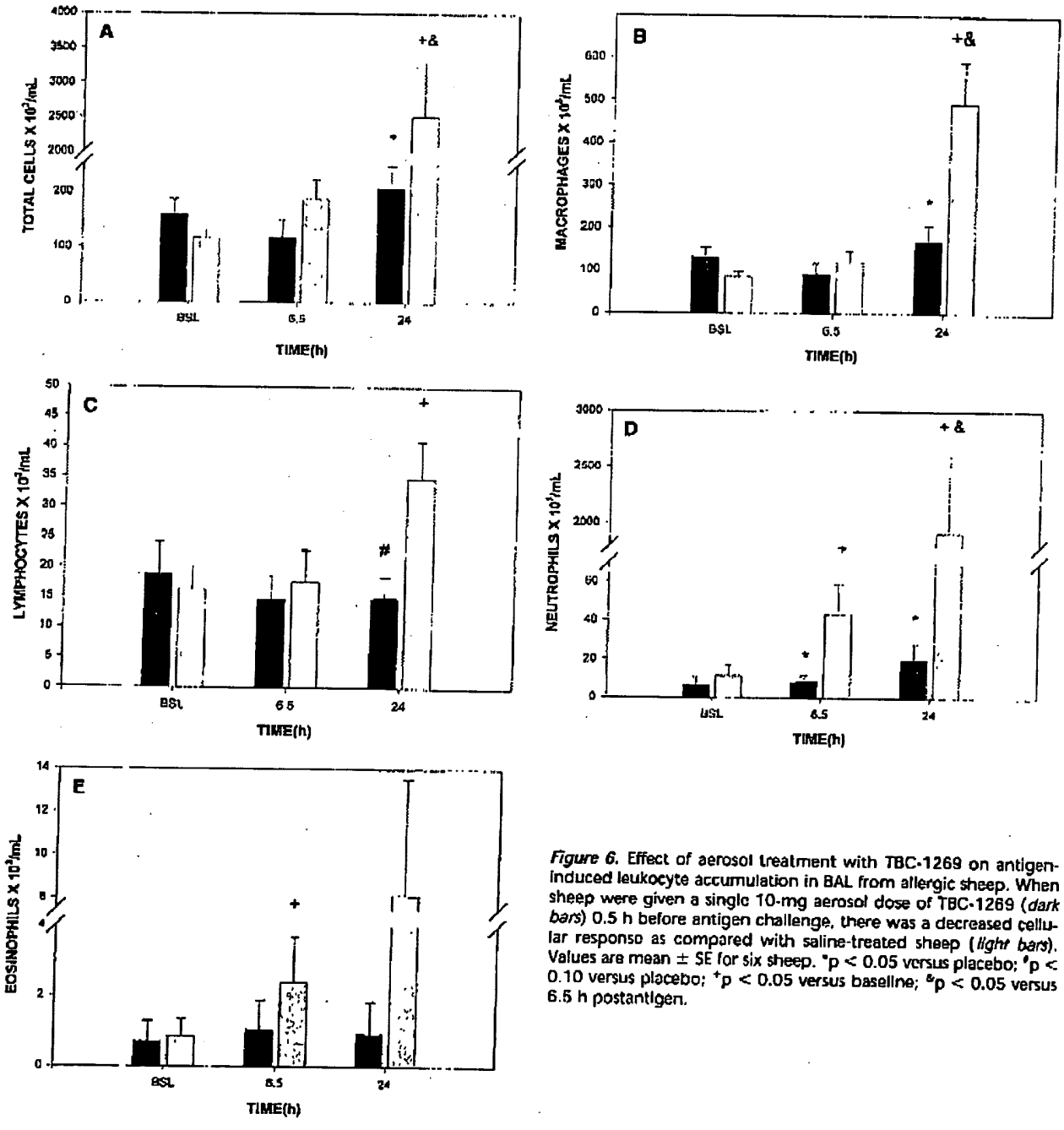

Figure 6. Effect of aerosol treatment with TBC-1269 on antigen-induced leukocyte accumulation in BAL from allergic sheep. When sheep were given a single 10-mg aerosol dose of TBC-1269 (*dark bars*) 0.5 h before antigen challenge, there was a decreased cellular response as compared with saline-treated sheep (*light bars*). Values are mean ± SE for six sheep. *$p < 0.05$ versus placebo; #$p < 0.10$ versus placebo; +$p < 0.05$ versus baseline; &$p < 0.05$ versus 6.5 h postantigen.

lenge provided that the time of treatment was in close proximity to the challenge. Figure 3 shows the effects of 10 mg of TBC-1269 given by aerosol either 90 min or 240 min after antigen challenge. Treatment at 90 min postchallenge effectively inhibited both LAR and the 24-h AHR, but the protective effect on the LAR and the 24-h AHR was lost if the compound was administered 240 min after challenge.

The collective data from the *in vitro* studies included in Table 2, the increased *in vivo* efficacy of TBC-1269 as compared with TBC-265, and the ability of both molecules to reduce the EAR make it likely that the primary target of these molecules is either P- or L-selectin, rather than E-selectin, which is maximally expressed 2 to 4 h after the initiation of the inflammatory response. To investigate this further, we next tested a small molecule selectin binding inhibitor that was 2- to 3-fold more effective at blocking L-selectin than either P- or E-selectin binding to sialyl-Lewis (sLe^x) glycolipids (Table 2). As expected, based on the relative molecular mass (M_r) and *in vitro* efficacy data, both a 5-mg and a 2.5-mg dose of MBPA aerosol administered 30 min before challenge gave a profile similar to TBC-1269, significantly inhibiting all three allergen-induced responses (Figures 4A and 4B, and Table 1). The protection was lost if the dose of MBPA was reduced to 1 mg (Table 1).

Although the data with the small molecule inhibitors suggest that L-selectin is the primary target for these small molecules, more conclusive evidence is provided by the last series of studies where the sheep were treated with the anti-sheep L-selectin antibody, DU1-29. As expected from the data with the small molecule inhibitors, 10 mg DU1-29 given 30 min before challenge as an aerosol significantly reduced the EAR and completely blocked the LAR and AHR after antigen challenge (Figures 5A and 5B). Treatment with a control antibody, MD6, had no effect on these parameters (Table 1).

Anti-inflammatory Activity

Figure 6 illustrates the anti-inflammatory capacity of TBC-1269. Pretreatment with 10 mg TBC-1269 resulted in an overall decrease in the total number of recoverable cells/ml in BAL ($p = 0.013$), neutrophils/ml ($p = 0.004$), and macrophages/ml ($p = 0.014$). Numbers of lymphocyte and eosinophils were also reduced, but overall, these changes did not achieve statistical significance. Although most cell types showed differences between drug and placebo 24 h after challenge, the neutrophil response was significantly ($p < 0.05$) suppressed at 6.5 h and 24 h after challenge in the treatment trial, when compared with the placebo trial.

Consistent with the reduction in the cellular response, there was also a reduction in the tissue kallikrein activity in BAL from the treated animals. Pretreatment with TBC-1269 resulted in a significant overall decrease ($p = 0.007$) in BAL tissue kallikrein levels compared with the placebo trial (Figure 7).

Histamine Release

The reduction of the peak EAR observed after pretreatment with TBC-1269 indicated that the compound may provide some inhibitory effect on mast cell mediator release. Analysis of BAL histamine levels at 30 min after challenge only showed detectable increases (> 1 nM) in two of the six controls. None of the treated animals had detectable concentrations at this time. One hour after challenge, histamine levels were increased in four of the six sheep in the placebo trial, whereas none of the animals in the treatment trial showed detectable increases. Median histamine levels were 1 nM for both groups before challenge. One hour after challenge, median histamine levels increased to 20.7 nM (range 1 to 100 nM) in the control trial, whereas in the treatment trial, the values remained at 1 nM (range 0; $p = 0.0375$).

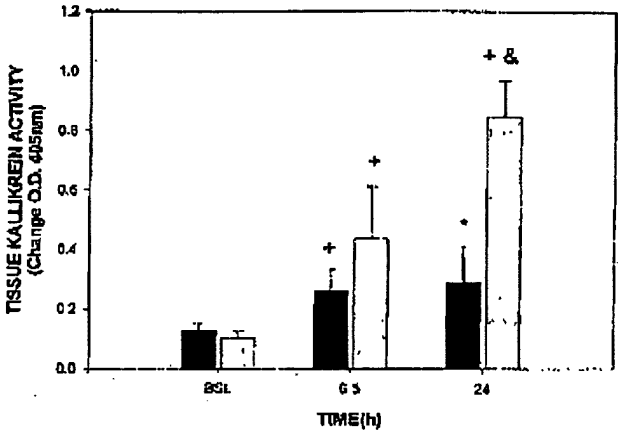

Figure 7. Effect of aerosol treatment with TBC-1269 on antigen-induced increases in BAL tissue kallikrein. When sheep were given a single 10-mg aerosol dose of TBC-1269 (*dark bars*) 0.5 h before antigen, there was a decreased response as compared with the saline-treated control animals (*light bars*). $^*p < 0.05$ versus placebo; $^+p < 0.05$ versus baseline; $^\&p < 0.05$ versus 6.5 h postantigen.

DISCUSSION

The results of this study provide novel evidence that small molecule selectin binding inhibitors can significantly reduce the EAR, the LAR, and the 24-h AHR that are induced by antigen challenge in allergic sheep. Specifically, this protective effect appears to be a function of inhibiting L-selectin because the nonoligosaccharide sLe^x mimetic, TBC-1269, and the smaller carbohydrate-free compound, MBPA, are more potent L- than E-selectin inhibitors and because this protection can be reproduced with an anti-L-selectin antibody. The additional evidence showing a reduction in histamine release during the EAR with TBC-1269 provides novel evidence that L-selectin binding may also influence mediator release.

Earlier studies have indicated that selectins are involved in allergic inflammation (2, 8, 17). Circulating E-selectin levels were significantly raised in patients with acute asthma when compared with concentrations in patients with stable asthma, atopic normal, or nonatopic normal volunteers (17). E-selectin was detected in biopsy specimens by immunolocalization in the bronchial submucosa of asthmatic subjects with airflow limitation (18), and was increased in skin biopsies from allergic subjects taken 3 to 6 h after intradermal injection of specific antigen. This expression correlated with the development of inflammatory cell infiltrates (8). Likewise, airway allergen challenge in primates resulted in increased expression of E-selectin exclusively on vascular endothelium 6 h after challenge (2), at which time the animals had an increase in BAL neutrophils and a LAR. The antigen-induced LAR and the neutrophil influx were blocked by pretreating the primates with an anti-E-selectin antibody. The effect of blocking E-selectin on post-antigen-induced AHR was not studied in these primates, but was examined in mice where P-selectin-deficient mice, sensitized to ovalbumin were found to exhibit less airway responsiveness and cell trafficking following ovalbumin challenge than did wild-type mice (19). The results of the present study

extend the previous findings by suggesting that L-selectin plays an important role in modulating antigen-induced responses. The protection against these physiologic endpoints obtained with lower doses of TBC-1269 and MBPA (better L- than E-selectin inhibitors) as compared with TBC-265 (better E- than L-selectin inhibitor) suggests that L-selectin is the primary target for these small molecules when given before antigen challenge. That similar results were obtained after treatment with the anti-sheep L-selectin antibody, DU1-29, confirms the role of L-selectin in these events. These data, however, do not rule out the possible contributions of E- and/or P-selectin. For example, the experiments showing that TBC-1269 was effective in blocking the LAR and the AHR when given 90 min after antigen challenge, but not 240 min after antigen challenge, are consistent with the molecule's inhibitory profile (i.e., E-selectin blocker) and the time course of E-selectin protein expression. The experiments showing protective effects of TBC-1269, when given 90 min after challenge do, however, demonstrate that inhibition of the LAR and AHR with TBC-1269 is not dependent on a reduction in the early antigen-induced response (*see* Figures 3A and 3B).

The ability of DU1-29 and TBC-1269, when given before antigen challenge, to modify the EAR, provides new data on the putative role of L-selectin in the modulation of allergic airway responses. That inhibition of L-selectin binding can reduce acute antigen-induced cell activation is new, but is consistent with previous findings in this animal model using anti-VLA-4 inhibitors (7). Based on the physiologic response to antigen in the presence of these L-selectin inhibitors, one could speculate that binding of L-selectin affects signal transduction in a way that reduces the initial cellular response to antigen. The finding that BAL histamine levels obtained during the EAR were reduced in treated animals supports this hypothesis.

The lavage data obtained with and without TBC-1269 pretreatment confirm the inhibitory effect on recruited leukocytes with the treated animals showing a reduction in the numbers of inflammatory cells recovered in BAL. Our result of decreased neutrophil numbers at 6.5 and 24 h after challenge confirms and extends the findings with primates using an E-selectin antibody. Our current results support our previous findings in sheep in which we demonstrated that the development of the LAR and AHR is dependent on the influx of activated granulocytes into the airways (20). Our previous work has also shown that increases in BAL tissue kallikrein activity are associated with airway inflammation during the LAR and the post-antigen-induced AHR (15). Here, we show for the first time that a selectin inhibitor blocks the antigen-induced increases in tissue kallikrein activity in BAL, a finding consistent with the anti-inflammatory activity of these molecules.

Although present upon the surfaces of leukocytes rather than endothelial cells, the role of L-selectin in the development of allergen-induced response may be quite similar to that hypothesized for E-selectin. In fact, because sLe^x-modified L-selectin has been shown to be a ligand for E-selectin (21), it is possible that blockade of L-selectin might also result in blockade of E-selectin under some circumstances. However, since E-selectin has also been shown to bind to other glycoprotein ligands, this dual selectin blockade would probably be a disease- and tissue-specific phenomenon. Nevertheless, our results indicate that the physiologic abnormalities indicative of asthma can be modulated by inhibiting the binding of either L- or E-selectin to their natural cell-associated ligands.

The results of this study provide the first evidence that aerosol administration of an L-selectin antibody or small molecule selectin inhibitors provides adequate protection against antigen-induced airway responses. Previous studies in this model have demonstrated similar efficacy using aerosol delivery for both monoclonal antibodies (4) and small molecule inhibitors to VLA-4 (7). The results of this study confirm and extend these previous observations and provide further evidence that local administration of such agents is a viable route for therapeutic administration of this class of compounds.

It is important to note that, while blocking effects of these agents are important to establish the role of selectins in allergic responses, the data in Table 1 showing dose-dependent effects of the different compounds on the physiologic responses, as well as the inability of the control antibody (MD6) to block the EAR, LAR, and AHR, are important experiments as well. These negative studies indicate that appropriate drug levels of active compounds must be achieved for the desired effect and that inactive compounds do not give false-positive responses in the model.

In summary, we have presented evidence that blockade of selectin binding can prevent the pathophysiological responses to allergen inhalation. These findings suggest that blockade of L-selectin may provide the basis for a novel therapy to control the acute pulmonary inflammatory response in experimental asthma.

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Effects of a selective phosphodiesterase IV inhibitor (CDP-840) in a leukotriene-dependent non-human primate model of allergic asthma

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Abstract: The activity of CDP-840, a novel, selective phosphodiesterase IV inhibitor was determined in a leukotriene-dependent non-human primate model of allergic asthma. Measurements of specific airway resistance (*s*Raw) were recorded in a dual chamber plethysmograph for 1 h and 3–5 h after challenge of allergic conscious squirrel monkeys with an aerosol of ascaris antigen. Orally administered CDP-840 (10 mg/kg; 1 h before challenge) produced partial inhibition (41 and 45%, respectively) of both the acute (1 h post antigen) response and the late (3–5 h post antigen) response to antigen but failed to alter the response to an aerosol of leukotriene D_4. In a second series of experiments, intravenous CDP-840 (5 mg/kg; 30 min before challenge) showed improved potency, producing 82% inhibition of the early and 51% inhibition of the late phase response. CDP-840 was inactive when tested intravenously at 1 mg/kg and was inactive against the 3–5 h response when administered after the early phase response (5 mg/kg; i.v. 60 min post antigen challenge). The novel phosphodiesterase IV inhibitor CDP-840 selectively inhibited antigen-induced bronchoconstriction in conscious squirrel monkeys. This effect appears to be independent of any direct bronchodilator action. It is concluded that the activity of CDP-840 in this model may be due to an inhibitory effect on mediator (e.g., leukotriene) release.

Key words: selective phosphodiesterase IV inhibitor, CDP-840, antigen-induced bronchoconstriction, non-human primate.

Résumé : L'activité du CDP-840, un nouvel inhibiteur sélectif de la phosphodiestérase IV, a été déterminée dans un modèle d'asthme allergique dépendant des leucotriènes chez des primates non humains. Des mesures spécifiques de la résistance des voies aériennes (*Rva*) ont été faites dans un pléthysmographe à deux chambres, pendant 1 h et 3–5 h après une provocation par un aérosol d'antigène ascaris chez des singes écureuils conscients, allergiques. Le CDP-840, administré par voie orale (10 mg/kg; 1 h pré-antigène), a provoqué l'inhibition partielle (41 et 45%, respectivement) tant de la réponse aiguë (1 h post-antigène) que de la réponse tardive (3–5 h post-antigène) à l'antigène, mais n'a pu modifier la réponse à un aérosol de leucotriène D_4. Dans une seconde série d'expériences, le CDP-840, administré par voie intraveineuse (5 mg/kg; 30 min pré-antigène) a montré une plus grande puissance, induisant une inhibition de 82% de la réponse en phase initiale et 51% de la réponse en phase tardive. Le CDP-840 a été inactif lorsque testé par voie intraveineuse à 1 mg/kg, et il a été inactif contre la réponse 3–5 h lorsqu'administré après la phase initiale (5 mg/kg; i.v. 60 min après la provocation antigénique). Le nouvel inhibiteur de phosphodiestérase IV, CDP-840, a inhibé de manière sélective la bronchoconstriction induite par un antigène chez des singes écureuils conscients. Il semblerait que cet effet soit indépendant d'une action bronchodilatatrice directe. On conclut que l'activité de CDP-840 dans ce modèle pourrait être due à un effet inhibiteur sur la libération de médiateurs (p. ex., les leucotriènes).

Mots clés : inhibiteur sélectif de la phosphodiestérase IV, CDP-840, bronchoconstriction induite par un antigène, primate non humain.
[Traduit par la Rédaction]

Introduction

Antigen-induced acute and late phase responses, airways hyperresponsiveness (AHR), and pulmonary inflammation are events that are consistently associated with atopic asthma (Barnes 1989). It is now generally accepted that the presence of chronic inflammation in the airways exacerbates the responses to allergic and non-allergic stimuli by altering the inherent responsiveness of lung tissues. The clinical efficacy of anti-inflammatory corticosteroids provides strong support for the importance of underlying inflammatory processes in the pathophysiology of asthma. Studies in a variety of animal models suggest that selective phosphodiesterase (PDE) IV inhibitors may have steroid-like actions and thus may prove beneficial in the therapy of asthma (Torphy and Undem 1991; Gozzard et al. 1996). Information from a variety of in vitro

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(Giembycz and Dent 1992; Qian et al. 1994; Hughes et al. 1996) and in vivo (Underwood et al. 1993, 1994; Holbrook et al. 1996; Hughes et al. 1995, 1996) animal studies with both selective and non-selective phosphodiesterase inhibitors support a role for the PDE IV isozyme as an important therapeutic target.

A major drawback in this area of drug development is the absence of esearch animals with clinical asthma per se. However, there are a number of models of asthma that have been developed in non-human primates (Patterson and Harris 1978; Patterson et al. 1988; McFarlane et al. 1987; Turner et al. 1994). These non-human primate models are particularly useful since they display distinct characteristics that, in many ways, mimic those observed in atopic human asthmatics. For example, ascaris-sensitive squirrel monkeys (*Saimiri sciureus*) have been important in the development of drugs that modify the synthesis and actions of leukotrienes (Brideau et al. 1991; Jones et al. 1989, 1995). We have recently extended our studies in this model and have established a non-invasive, double plethysmographic technique for studying the leukotriene-dependent early (E) and late (L) phase bronchoconstriction to aerosolized ascaris antigen (McFarlane et al. 1994; Jones et al. 1995). Immediate bronchoconstriction and late phase obstructions are characteristic features of atopic asthmatics, and there is now overwhelming evidence for the involvement of leukotrienes in these responses (Creticos et al. 1984; Tagari et al. 1990; Manning et al. 1990; Rasmussen et al. 1992). The objective of the present study was to examine the activity of CDP-840 (Hughes et al. 1996), a novel, selective PDE IV inhibitor in this leukotriene-dependent non-human primate model of allergic asthma.

Methods

Animal care

All animals and procedures employed in these studies were conducted in strict accordance with the principles and guidelines of the Canadian Council on Animal Care and with the approval of the institutional Animal Care Committee.

Supply and training of squirrel monkeys

Male squirrel monkeys (*Saimiri sciureus*), weight range 1.0–1.2 kg, were obtained from the Primate Import Corp., Fort Washington, NY (Charles River Research Primate Corporation). The animals were in good health and free of parasitic infections, and all had negative tests to tuberculoprotein. The monkeys were trained to sit in restraining chairs, which allowed complete freedom of the hands and legs. The chair was constructed of metal, equipped with a vertically adjustable seat and a horizontally mounted plastic plate with a hole 80 mm in diameter, through which the head of the animal protruded. A second adjustable plate, shaped to fit the contour of the neck, was mounted on the horizontal plate and served as a collar to prevent the monkeys from pulling their head through the hole while in the sitting position. In addition, a loosely fitted curved head holder (180 × 40 mm) lined with foam rubber (15 mm thick) was mounted on the back of the chair and served to support and cushion the animal's head. Animals were conditioned over a period of several weeks to sit in the chair and breathe in a cylindrically shaped face mask, after which they were placed into an exposure chamber for adaptation to the manipulative procedures for drug and aerosol administration.

Screening protocol for skin reactivity

The monkeys used were initially cutaneously screened for sensitivity to *Ascaris suum* antigen with dilutions of the antigen extract containing approximately 220 000 protein nitrogen units/mL (PNU/mL). For assessment of skin reactivity to intradermal injection of dilutions of *Ascaris suum* extract, monkeys were anesthetized with ketamine hydrochloride, 25 mg/kg i.m. The animal was placed in a supine position, and abdominal and chest areas were shaved and washed. A volume of 0.5 mL/kg of Evans blue dye, 0.5%, was injected intravenously, and a period of about 5 to 10 min was allowed before intradermal injections were started. Dilutions of antigen of 1:10, 1:100, 1:1000, 1 : 10 000, 1 : 100 000, and 1 : 1 000 000 and saline as control were made. A total volume of 50 µL of antigen was injected intradermally at a different site for each dilution. Reactions were recorded in millimetres and arbitrarily scored at 10 and 30 min. Monkeys with positive skin reactivity at dilutions of at least 1:1000 were used for pulmonary function studies.

Measurement of specific airway resistance (*s*Raw)

The apparatus used consisted of a dual rectangular thoracic plethysmograph and a cylindrically shaped nasal plethysmograph developed in collaboration with Buxco Electronics Inc. (Sharon, Conn.). The thoracic plethysmograph was constructed of 1.25 cm transparent polycarbonate with inside dimensions 35 cm high × 27.5 cm long × 17.5 cm deep with a dead-space volume of 11.8 L. The nasal plethysmograph was made of clear acrylic plastic and measured 19.4 cm long with an inside diameter of 5 cm and a dead space of 0.4 L and was hooked up to a sealed accessory chamber of 11.4 L via a side arm. Each plethysmograph was equipped with ports for a calibrating syringe, a bias air flow pump with long inlet tubing of high resistance (Buxco Electronics Inc., Sharon, Conn.), a pneumotachograph, 325 mesh, stainless-steel screens (Buxco Electronics Inc.), a high sensitivity Validyne transducer, model DP-45-14, range ± 2 cmH_2O (Validyne Engineering Corp., Northidge, Calif.), and airtight latex-rubber nasal and neck diaphragm seals. In addition, a fast response digital temperature–humidiguide, Taylor, model 5566 (Comeau Technique, Montréal, Que.), was mounted on the door of the thoracic chamber to continuously monitor changes in temperature and humicity inside the thoracic chamber. A separate digital temperature probe, model THK-6000, type-K (Comeau technique), was inserted into the nasal plethysmograph to register temperature changes within the chamber. The nasal plethysmograph was also equipped with a port that was opened for aerosol delivery of drugs via a T-shaped valve but was closed during specific airway resistance measurements. Acrosolized solutions were generated (1–5 µm particle size) by a DeVilbiss ultrasonic nebulizer, model 25C (The DeVilbiss Co., Somerset, Pa.), and were delivered into the nasal chamber at a flow rate of 2 L/min for 5 min with the aid of a DeVilbiss Pulmo-Aide pump, model 561 series. A non-rebreathing valve plastic ball (1.5 g) located on the nasal plethysmograph opened when the monkey exhaled and closed on inhalation, rhythmically, during aerosol administration. In addition, two built-in T-shaped valves in front of the nasal pneumotachograph and the Validyne transducer, respectively, were closed during aerosolization to prevent aerosol particles from entering these devices. Similarly, a built-in valve in the arm connecting the nasal chamber to the accessory-volume chamber was closed during aerosol delivery. The concentration of CO_2 in the expired breath of the monkeys was measured continuously with a Beckman Instrument LB-2 Medical Gas Analyzer (Summit Technologies Inc., Toronto, Ont.) from a port in the nasal plethysmograph in order to monitor excessive buildup of CO_2 and the possibility of rebreathing.

To measure thoracic and nasal volume changes, the trained conscious squirrel monkeys were seated in the volume-sensitive thoracic plethysmograph with their head protruding though the fitted latex rubber collar, which formed an airtight seal around the neck. The door to the chamber, which was equipped with a 0.625-cm rubber gasket, was closed to form an airtight seal. The head of the monkey was supported in the cushioned head holder while its muzzle was inserted into the nasal plethysmograph through an appropriate size circular

Table 1. Profile of skin reactivity to *Ascaris suum* antigen in squirrel monkeys.

Monkey No.	Skin reactivity (antigen dilution)					
	1 : 10	1 : 100	1 : 1000	1 : 10 000	1 : 100 000	1 : 1 000 000
61	+++	+++	+++	+++	++	+
62	+++	+++	+++	++	++	+
63	+++++	+++++	++++	+++	++	++
65	++++	++	+++	±	±	±
66	++++	+++	+++	+	+	+
67	++++	+++	+++	+++	++	++
74	++++	+++	+++	±	++	+
79	++++	+++	+	+	++	+
80	+++	++	+	±	±	±
91	+++	++	+	±	±	±

Note: For assessment of skin reactivity to intradermal injection of *Ascaris suum* extract (containing 220 000 protein nitrogen units per millilitre), monkeys were anesthetized with ketamine hydrochloride, 25 mg/kg, im. The animal was placed in a supine position, and abdominal and chest areas were shaved and washed. A volume of 0.5 mL/kg of Evans blue dye, 0.5%, was injected intravenously, and a period of about 5 to 10 min was allowed before intradermal injections were started. Reaction to 50 μL of diluted extract at 30 min was scored accordingly: +++++, very intense purple wheal, >15 mm diameter; ++++, very intense blue wheal, >10 mm diameter; +++, intense blue wheal, 5–10 mm diameter; ++, medium response, 5 mm diameter; +, weak response, <5 mm diameter; ±, very weak response, just visible; –, no blueing response observed.

hole in a fixed latex-rubber diaphragm, 0.125 cm thick (Small Parts, Miami, Fla.), to form an airtight seal.

Flow in both plethysmographs was measured with high sensitivity Validyne transducers (range ± 2 cmH_2O) and preamplified. The preamplified signals were fed into a Buxco Electronics non-invasive respiratory analyzer, model LS-20, and eight derivations of respiratory function were made, namely, peak expiratory flow rate, tidal volume, respiratory rate, minute volume, inspiratory time, expiratory time, relaxation time (time in milliseconds to reach 30% residual volume), and specific airway resistance, all on a breath by breath basis. The derivations were fed into a data logger for tabulated printout as well as into a personal computer (386PC-25). In addition, the thoracic and nasal wave forms were displayed continuously on a scrolling monitor screen (Hewlett Packard Vectra 286). The respiratory analyzer measured the time delay between the thoracic flow and the nasal plethysmograph flow (the lag time between the two wave forms) at the transition from inspiration to expiration and derived a value for specific airways resistance (*sRaw*). *sRaw* is defined as airways resistance × thoracic gas volume expressed in $cmH_2O{\cdot}s$.

Assessment of early and late phase *sRaw* control responses

For assessment of early and late phase *sRaw* control responses, the monkeys were challenged with an aerosol of *Ascaris suum* antigen, 1:25 dilution, at intervals of no less than 3 weeks. Antigen aerosol of particle mist, 1 to 5 μm minimum size, was generated by a DeVilbiss ultrasonic nebulizer, and delivered as described above for a period of 15 min. Following challenge, the time course and changes in specific airway resistance for the early phase were recorded at 1-min intervals for a duration of 1 h post challenge. At this time point recording was stopped and the monkey was removed from the plethysmograph apparatus and returned to its home cage. Two hours later, i.e., 3 h post antigen challenge, the monkey was returned to the plethysmograph to continue recording for the development of late phase responses for an additional 2 h. A limited number of experiments were carried out on four animals, which were kept in the recording chamber for a period of 2.5 h post antigen challenge. These experiments were technically difficult to perform since most animals do not sit quietly in the recording chamber for this length of time. However, these experiments were designed to determine whether there was a clear demarcation point between the early and late phase responses.

Drug testing protocol

Ten trained squirrel monkeys with reproducible *sRaw* responses to ascaris antigen were used to assess the inhibitory effects of CDP-840. On the test day, drug was dissolved in 1% methocel and administered orally to the monkeys at a dose of 10 mg/kg in a volume of 1 mL/kg body weight. One hour later the monkeys were challenged with ascaris antigen aerosol 1:25 dilution for 15 min. Immediately after challenge, the changes in the early phase *sRaw* responses were measured continuously for 1 h and after a 2-h rest period (no recording), the late phase *sRaw* responses were recorded for an additional 2 h. The effects of CDP-840 administered intravenously (5 and 1 mg/kg, 30 min pretreatment) were also determined in 5 squirrel monkeys, using a similar recording procedure. A fourth series of experiments was carried out in 3 animals, in which CDP-840 (5 mg/kg; 1 h post antigen) was administered intravenously at the end of the early phase (1 h post antigen). A fifth series of experiments was carried out in 3 animals challenged with an aerosol of leukotriene D_4 following oral treatment with CDP-840 (10 mg/kg; 1 h pretreatment).

Pharmacokinetic analysis of CDP-840

Drug levels were determined by taking venous blood (approximately 1 mL) from predosed animals into heparinized tubes and then centrifuging to provide plasma (approximately 0.3 mL). Aliquots of plasma were diluted with an equal volume of acetonitrile and centrifuged to remove protein precipitate. The supernatant was injected directly into a C-18 HPLC column with UV detection. Quantitation was done relative to a clean (control) blood sample spiked with a known quantity of drug. Detection limits were approximately 0.1 μg/mL.

Materials

CDP-840 (*R*-(+)-4-[2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl]-pyridine) was synthesized in the department of Medicinal Chemistry, Celltech Therapeutics Ltd., and was dissolved in 1% methocel for oral administration and sterile saline for i.v. administration. *Ascaris suum* extract (220 000 protein nitrogen units/mL) was obtained from Greer Laboratories Inc., Lenoir, N.C., and was diluted in sterile 0.9% NaCl (1:25).

Table 2. Inhibition of ascaris-induced bronchoconstriction in conscious squirrel monkeys.

(A) Pleural catheter technique.

Compound	Dose (mg/kg p.o.)	n	% inhibition R_L Early	Late	Reference
L-691,816 (5-LO inhibitor)	1 mg/kg	5	79	nd	Hutchinson et al. 1993
L-651,392 (5-LO inhibitor)	5 mg/kg	5	71	58.4	McFarlane et al. 1987
MK-886 (FLAP inhibitor)	1 mg/kg	5	85	nd	Gillard et al. 1989
MK-591 (FLAP inhibitor)	1 mg/kg	5	71	nd	Brideau et al. 1991
MK-571 (Cys LT_1 antagonist)	0.5 mg/kg	5	74	nd	Jones et al. 1989

(B) Dual plethysmographic technique.

Compound	Dose (mg/kg p.o.)	n	% inhibition sRaw Early	Late	Reference
Montelukast (Cys LT_1 antagonist)	0.1 mg/kg	5	69	64	Jones et al. 1995

Note: The methodology for the pleural catheter technique was originally described in McFarlane et al. (1987). All data in the above table were obtained in previous studies (see reference listed). nd, not determined.

Table 3. Baseline pulmonary function values for conscious squirrel monkeys measured by a non-invasive plethysmographic technique.

Parameter of pulmonary function	Unit	Mean value ± SEM ($n = 10$)
Peak expiratory flow rate (PEFR)	mL/s	48.0±3.1
Tidal volume (V_T)	mL	15.3±0.9
Respiratory rate (RR)	breaths/min	62.7±4.1
Minute volume (MV)	mL	984.4±103.4
Inspiratory time (IT)	s	0.44±0.03
Expiratory time (ET)	s	0.68±0.06
Relaxation time (RT)	s	0.42±0.03
Specific airway resistance (sRaw)	$cmH_2O \times s$	4.7±0.2

Note: Values are mean baseline values ± SEM of 3–5 controls for 10 conscious squirrel monkeys. All values are 5-min averages obtained for 30 min before challenge with ascaris antigen.

Results

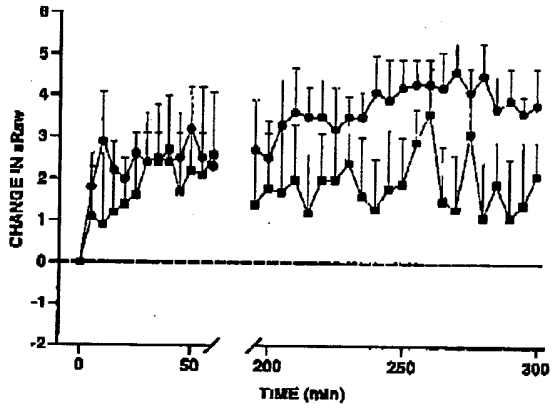
Skin reactivity

All animals selected for lung challenge were skin test positive (Table 1). All of these animals responded with an early and a late response to ascaris aerosol. The magnitude of the airway response could not be reliably predicted by the magnitude of the skin response. For example, squirrel monkeys 80 and 74 gave the strongest lung response to ascaris challenge but, although skin test positive, were not necessarily the most skin reactive. All animals used to obtain baseline measurements and to assess the activity of CDP-840 (Table 1) were skin and lung reactive to ascaris.

Dual plethysmographic technique for recording sRaw

Previous studies in this animal species have demonstrated that leukotriene receptor antagonists (Jones et al. 1989; 1991), 5-lipoxygenase (5-LO) inhibitors (McFarlane et al. 1987), and 5-lipoxygenase activating protein (FLAP) inhibitors (Gillard et al. 1989; Brideau et al. 1991) are potent inhibitors of the acute bronchoconstrictor response to antigen challenge (Table 2). These studies were carried out using a pleural catheter technique in order to obtain a transpulmonary pressure measurement used in the calculation of lung resistance (R_L) and dynamic compliance (Cdyn). This invasive technique has since been replaced by the less invasive, dual plethysmographic technique (Buxco Electronics Inc.), described here, which allows one to determine both early and late phase measurements of changes in sRaw in response to ascaris challenge. Baseline pulmonary function values for conscious squirrel monkeys measured by this non-invasive plethysmographic technique are summarized in Table 3. Results obtained with montelukast, a selective Cys LT_1 receptor antagonist, using this dual plethysmograph are included in Table 2. These findings are consistent with previous results and indicate that leukotrienes are involved in both the early and the late phase response to allergen challenge.

Fig. 1. Effect of CDP-840 (10 mg/kg p.o. 1 h before challenge; ■, $n = 5$) on control (●; $n = 5$) antigen-induced early and late phase increases in specific airway resistance (sRaw) in conscious squirrel monkeys. Each point represents the mean ± SEM of 5 values expressed in absolute sRaw values. Animals were removed from the recording chamber from time 60 min to 180 min post ascaris challenge.



Effects of CDP-840 versus ascaris antigen induced bronchoconstriction

In control experiments, inhaled antigen produced an acute bronchoconstriction, which increased sRaw by approximately

Table 4. Effect of CDP-840 versus ascaris-induced bronchoconstriction in conscious squirrel monkeys.

	n	Baseline *s*Raw	Early phase Δ *s*Raw	Late phase Δ *s*Raw	% inhibition	
					Early phase	Late phase
Series 1						
(SM 61, 66, 67, 74, 91)						
Control	5	3.90±0.40	2.44±0.35	3.50±0.74		
CDP-840, 10 mg/kg p.o. (1 h pre)	5	4.45±0.69	1.85±1.52	1.72±0.99	41	45
Series 2						
(SM 61, 65, 74, 80, 91)						
Control	5	4.81±0.35	2.67±0.53	3.37±0.47		
CDP-840, 5 mg/kg i.v. (30 min pre)	5	5.32±0.86	0.29±0.13	1.36±0.92	82*	51*
Series 3						
(SM 62, 63, 67, 74, 79, 91)						
Control	6	4.79±0.48	2.40±0.31	2.79±0.27		
CDP-840, 1 mg/kg i.v. (30 min pre)	6	5.43±0.89	1.73±0.46	1.96±0.68	32	38

Note: All *s*Raw values are means ± SEM. *n*, number of monkeys used for each study. SM numbers refer to the specific monkeys used for each study (from Table 1). Δ *s*Raw is the average change over 60 min post ascaris challenge (early phase) and 180–300 min post ascaris challenge (late phase).
*$p < 0.01$.

Fig. 2. Plasma levels of CDP-840 in conscious squirrel monkeys administered 10 mg/kg CDP-840 p.o. (●; $n = 3$) and 5 mg/kg CDP-840 i.v. (■; $n = 3$). Each point represents the mean ± SEM plasma concentration at each time point after drug administration. In the above study only trace amounts (<0.1 μg/mL) of the N-oxide metabolite (equiactive to CDP-840 in vitro) were detected.

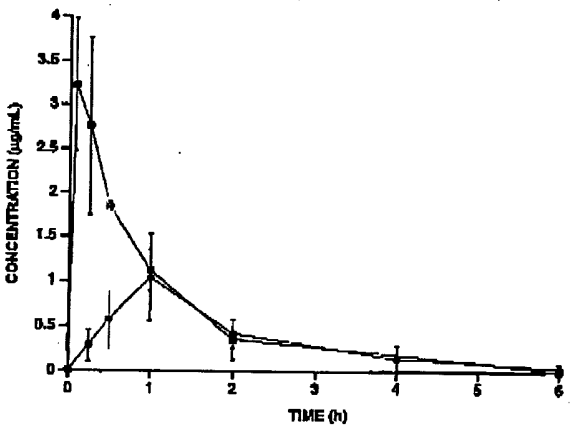
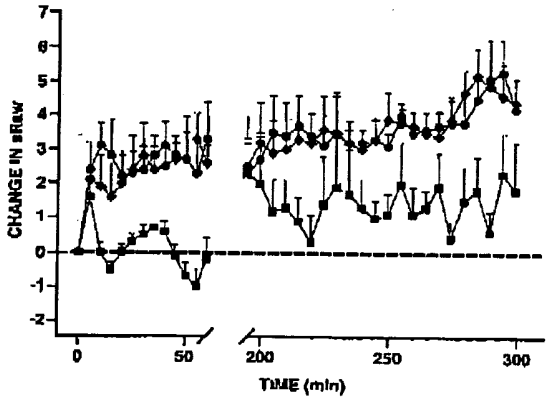


Fig. 3. Effect of CDP-840 (5 mg/kg i.v. 30 min before challenge; ■; $n = 5$) and 0.9% NaCl (1 mL/kg i.v. 30 min before challenge; ◆; $n = 5$) on control (●; $n = 5$) antigen-induced early and late phase increases in specific airway resistance (*s*Raw) in conscious squirrel monkeys. Each point represents the mean ± SEM of 5 values expressed in absolute *s*Raw values. Animals were removed from the recording chamber from time 60 min to 180 min post ascaris challenge.



50–70%. The response was recorded for a period of 1 h, at which point the animals were returned to their home cage for a period of 2 h. After reestablishment in the test chamber, these animals continued to develop substantial bronchoconstriction, which was recorded for an additional 2 h period. In separate experiments ($n = 4$), recording the early phase response for 2.5 h post antigen challenge failed to uncover a clear separation between the early and late phases of bronchoconstriction. Pretreatment with CDP-840 (10 mg/kg; 1 h before challenge) inhibited the early and late response by 41 and 45%, respectively (Fig. 1 and Table 4). Analysis of the plasma levels of CDP-840 in 3 animals administered 10 mg/kg, orally, revealed a peak plasma level of approximately 1 μg/mL at 1 h post drug administration (Fig. 2).

A second series of experiments was carried out with CDP-840 (5 mg/kg; 30 min before challenge) administered intravenously. With this route of administration CDP-840 was markedly more potent against both the early and late phase responses, producing approximately 82 and 51% inhibition, respectively (Fig. 3 and Table 4). When administered at a dose of 1 mg/kg, CDP-840 was inactive in 6 animals (series 3; Table 4). Plasma levels at 30 min post drug administration (5 mg/kg i.v.) were approximately 2 μg/mL but were less than 0.3 μg/mL at 30 min post 1 mg/kg i.v. (Fig. 2).

A fourth series of experiments was carried out with CDP-840 (5 mg/kg) administered intravenously at the end of the early phase response (i.e., 1 h post antigen challenge). CDP-840 failed to block the response that developed 2 h later (Fig. 4).

In a fifth series of experiments CDP-840 (10 mg/kg; p.o. 1 h before challenge) failed to significantly alter the response to aerosol challenge with leukotriene D_4 (Fig. 5; $n = 3$).

Fig. 4. Effect of CDP-840 (5 mg/kg i.v., 1 h post antigen; ■; $n = 3$) on control (●, $n = 3$) antigen-induced early and late phase increases in specific airway resistance (*s*Raw) in conscious squirrel monkeys. Each point represents the mean ± SEM of 3 values expressed in absolute *s*Raw values. Animals were removed from the recording chamber from time 60 min to 180 min post ascaris challenge.

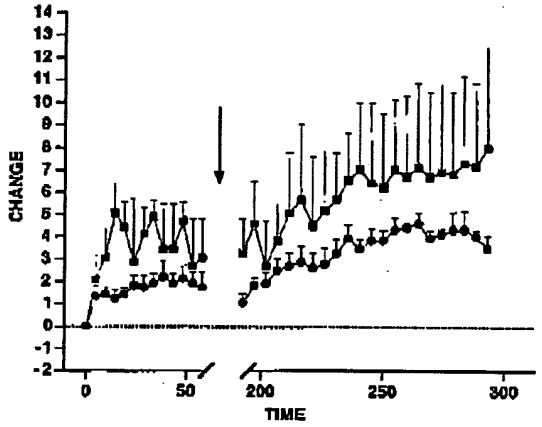
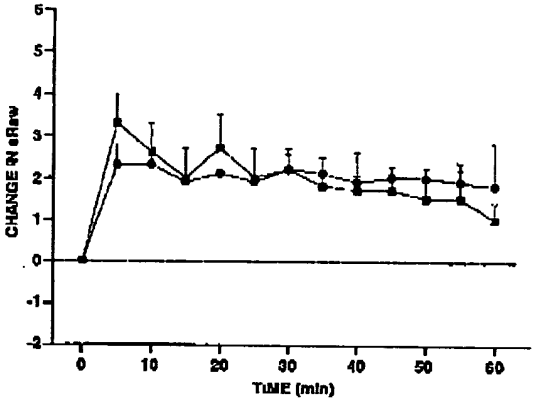


Fig. 5. Effect of CDP-840 (10 mg/kg p.o. 1 h before challenge; ■; $n = 3$) on control (●; $n = 3$) responses to an aerosol challenge with leukotriene D_4 (50 µg/mL; 10 min aerosol). Each point represents the mean ± SEM of 3 determinations expressed in absolute *s*Raw values.



Discussion

Positive clinical results with the latest generation of leukotriene receptor antagonists and leukotriene biosynthesis inhibitors have proven the importance of these lipid mediators in allergic and non-allergic asthma (Margolskee et al. 1991; Rasmussen et al. 1992; Spector et al. 1992). Ascaris-sensitive squirrel monkeys have been an important animal model for predicting and for studying leukotriene-dependent allergic asthma (Table 2; McFarlane et al. 1994; Jones et al. 1995). Thus, it follows that this animal model of asthma is well suited for determining the role played by PDE IV isozymes in the modulation of lung responses to these arachidonic acid metabolites. As in the past, animals used for lung studies were selected on the basis of their skin reactivity to ascaris. Skin test negative animals were always lung challenge negative to ascaris. It is impossible to draw any other conclusions regarding lung and skin reactivity since only a single dose of ascaris was used for the present lung function studies. This finding is consistent with previous results obtained in rhesus and cynomolgus monkeys (O'Neil and Goodman 1981).

In the present study, the novel and selective PDE IV inhibitor CDP-840 attenuated antigen-induced bronchoconstriction. These findings suggest a potential for this class of compounds in the therapy of allergic asthma in addition to their potential as anti-inflammatory agents. The inhibition was evident against both the acute early phase and the response that continued to develop 3–5 h after antigen challenge (late phase). CDP-840, although only moderately active following oral administration (as a result of variable absorption), was particularly effective following intravenous administration of 5 mg/kg (30 min before antigen challenge). This dose of CDP-840 produced average blood levels of 1.8–2.0 µg/mL immediately before challenge but had no significant effect on basal *s*Raw measurements. These findings indicate that CDP-840 exhibits anti-allergic activity at doses that failed to produce bronchodilatation. This hypothesis receives further support from separate studies in which orally administered CDP-840 (10 mg/kg) failed to alter bronchoconstriction induced by aerosolized leukotriene D_4. Preliminary observations indicate that PDE IV inhibitors when administered i.v. after the early phase allergic response failed to block the late response, further demonstrating that this compound is not simply a bronchodilator. These suggest that the activity of this drug is dependent on actions in the early phase.

Inhibition of antigen-induced acute bronchoconstriction with CDP-840 is consistent with recent findings in guinea pigs and rabbits with the PDE IV inhibitor rolipram (Hughes et al. 1996; Gozzard et al. 1996; Underwood et al. 1993). Responses to antigen challenge in these models are thought to be mediated by released leukotrienes in addition to biogenic amines such as histamine. Thus, the most likely mechanism of action for CDP-840 is inhibition of mediator release from mast cells. PDE IV inhibitors, acting through elevation of cAMP, are known to inhibit antigen-induced release of leukotrienes and to a lesser extent histamine release from guinea-pig lung (Heaslip et al. 1992). Results reported from studies in cynomolgus monkeys (Turner et al. 1994) are somewhat inconsistent with this interpretation, since rolipram failed to block the acute phase bronchoconstriction to antigen. Differences in the activity of anti-asthmatic agents in various primate models, however, are not unique to PDE IV inhibitors. For example, in addition to rolipram, the mast cell stabilizer disodium cromoglycate (Eady et al. 1985) and the leukotriene D4 receptor antagonist ICI-198615 (Patterson et al. 1988) did not completely block the early phase bronchoconstriction in cynomolgus or rhesus monkeys. In contrast, man and squirrel monkeys appear to be particularly sensitive to anti-leukotriene drugs. The reason for this is not known, but one interpretation is that some primates have a greater histamine component to their acute allergic lung response. Sodium cromoglycate and steroids are also known to be effective in man but have not been tested in squirrel monkeys. Mediator release from squirrel monkey lungs has not been studied, but one could speculate that leukotriene release from this primate species may be particularly sensitive to PDE IV inhibitors. There are some recent

data available which demonstrate that PDE IV inhibitors can block LTB_4 generation from chopped lungs and arachidonic acid release from human mononuclear cells (Hichami et al. 1995). It would be of particular interest to compare the activity of PDE IV inhibition on mediator release from isolated rhesus, cynomolgus, squirrel monkey and human lung.

The persistent effect of CDP-840 for up to 5 h post challenge is quite remarkable, given that there are no measurable blood levels at this time (limit of detection is 0.1 μg/mL). The response at 3–5 h has been called a late response and like the late phase in man is known to be leukotriene dependent. However, it is not known whether this response in squirrel monkeys is a true late response or whether it simply represents a continuation of the acute response. Results from experiments in which the early phase response was followed for 2.5 h failed to uncover an obvious separation between the two phases, supporting the latter interpretation. Eosinophils, T-cells, and specific cytokines have been shown to be associated with late responses in allergic animal models and in human asthma. Eosinophil migration and cytokine release are cAMP modulated events, which reportedly can be inhibited by PDE IV inhibitors. Turner et al. (1994) reported that rolipram inhibited TNFα release in bronchoalveolar lavage (BAL) 4 h post antigen challenge in ascaris-sensitive cynomolgus monkeys. It is not known whether similar mechanisms with a similar time course operate in ascaris-challenged squirrel monkeys. Preliminary results in which CDP-840 was inactive if administered after antigen challenge suggest that the response at 3–5 h is closely linked to events that occur in the early phase and as such may be confined to an effect on mast cells via inhibition of lipid mediator release.

In conclusion, the novel PDE IV inhibitor CDP-840 inhibited antigen-induced early and late phase bronchoconstriction in conscious squirrel monkeys. This effect was independent of any bronchodilator activity, suggesting that selective PDE IV inhibitors may prevent leukotriene release in response to antigen challenge of allergic non-human primates. Should this effect extend to man one would expect that this class of compounds would have a therapeutic benefit in human asthma.

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The Journal of Immunology

New IL-17 Family Members Promote Th1 or Th2 Responses in the Lung: In Vivo Function of the Novel Cytokine IL-25[1]

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We have biologically characterized two new members of the IL-17 cytokine family: IL-17F and IL-25. In contrast to conventional in vitro screening approaches, we have characterized the activity of these new molecules by direct in vivo analysis and have compared their function to that of other IL-17 family members. Intranasal administration of adenovirus expressing IL-17, IL-17C, or IL-17F resulted in bronchoalveolar lavage neutrophilia and inflammatory gene expression in the lung. In contrast, intranasal administration of IL-25-expressing adenovirus or IL-25 protein resulted in the production of IL-4, IL-5, IL-13, and eotaxin mRNA in the lung and marked eosinophilia in the bronchoalveolar lavage and lung tissue. Mice given intranasal IL-25 also developed epithelial cell hyperplasia, increased mucus secretion, and airway hyperreactivity. IL-25 gene expression was detected following *Aspergillus* and *Nippostrongylus* infection in the lung and gut, respectively. IL-25-induced eosinophilia required IL-5 and IL-13, but not IL-4 or T cells. Following IL-25 administration, the IL-5$^+$ staining cells were CD45R/B220$^+$, Thy-1$^{+/-}$, but were NK1.1-, Ly-6G(GR-1)-, CD4-, CD3-, and c-*kit*-negative. γ-common knockout mice did not develop eosinophilia in response to IL-25, nor were IL-5$^+$ cells detected. These findings suggest the existence of a previously unrecognized cell population that may initiate Th2-like responses by responding to IL-25 in vivo. Further, these data demonstrate the heterogeneity of function within the IL-17 cytokine family and suggest that IL-25 may be an important mediator of allergic disease via production of IL-4, IL-5, IL-13, and eotaxin. *The Journal of Immunology,* **2002, 169: 443–453.**

Interleukin-17 is a CD4$^+$ T cell-derived cytokine that promotes inflammatory responses in cell lines and is elevated in rheumatoid arthritis, asthma, multiple sclerosis, psoriasis, and transplant rejection. Human IL-17 exists as glycosylated 20- to 30-kDa homodimers. IL-17 was initially recognized for its similarity to a sequence belonging to *Herpesvirus saimiri*, but it had little relatedness to any other known cytokines or other mammalian proteins (1, 2). More recently, two additional members of the IL-17 family have been described: IL-17B and IL-17C (3, 4). Although all three family members may promote inflammation and hemopoiesis, some of the responses of IL-17B and C are distinct from those described for IL-17. For example, IL-17 has been shown to signal through the IL-17R molecule and promote production of TNF-α, IL-1β, IL-6, IL-8, and G-CSF (5–7). In contrast, IL-17B and C do not appear to bind to IL-17R and only promote expression of TNF-α and IL-1β in vitro (3). Recently, a human sequence of IL-17F has been described from lymphocytes and patients with asthma (8, 9). These reports also described the production of cytokines from cells cultured with IL-17F. Also, a human IL-25 sequence has recently been described with close homology to other members of the IL-17 family (10). These findings demonstrate that the currently identified IL-17 family members promote distinct responses and may bind a variety of receptors on different cell types. In this report, we describe the in vivo biology of IL-17C as well as two additional family members, IL-17F and IL-25.

In recent years, a significant number of novel genes have been identified in sequence databases by their homology to known cytokines. However, the function of molecules discovered in this manner can be difficult to determine unless it is very similar to a previously known homologue. Ectopic overexpression of novel genes in transgenic mice has proven to be a useful strategy for function determination, but is laborious and difficult to control; the presented phenotype can often represent a process quite distal to the primary function of the transgene. Based on previous work, we have developed an in vivo screening strategy for the function of novel cytokine homologues based upon ectopic expression in the lung following adenovirus (Ad)[5]-mediated gene transfer (11–13). Biological responses to the transferred gene are indicated by altered cell infiltration into the lung and/or by changes in mRNA levels, measured by real-time PCR, of a panel of cytokine, chemokine, and receptor genes. The lung is both a convenient organ for localized and efficient Ad infection and is one of the most reactive organs to immune and inflammatory stimuli. This latter

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[5] Abbreviations used in this paper: Ad, adenovirus; EST, expressed sequence tag; m, mouse; i.n., intranasally; BAL, bronchiolar lavage fluid; PAS, periodic acid-Schiff; PSSM, position-specific scoring matrix; h, human; γ_c, common γ.

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property makes ectopic expression in the lung an especially sensitive technique. We demonstrate that Ad infection of mouse lungs with the IL-17 family members IL-17C and IL-17F results in neutrophilia and inflammatory gene expression such as IL-6 and IFN-γ. In contrast, IL-25 Ad infection of the lung promotes responses similar to those mediated by Th2 cells, including IL-4, IL-5, IL-13, and eotaxin production, followed by eosinophil infiltrate, mucus production, and airway hyperreactivity.

Materials and Methods

Cloning of mouse and human IL-17 family members

IL-17B. GenBank database expressed sequence tag (EST) sequences (IMAGE clone 475876 mouse and 783987 human) were identified in an IL-17 computational screen. These clones were ordered (Research Genetics, Huntsville AL) and the inserts were completely sequenced yielding full-length cDNAs for IL-17B_Mu and IL-17B_Hu.

IL-17C. A partial EST clone (HTGED19R) was identified in a computational screen of the Human Genome Science (Rockville, MD) database by homology to IL-17 family members. Primers based on this partial sequence were used to screen a panel of human cDNA libraries. A clone was identified from a cDNA library of PBMC. The full-length cDNA was PCR amplified from this library using primers GTGTGGCCTCAGGTATAA GAG and CTAAGGCCCCACGGCCTTGG, cloned into the TOPO vector (Invitrogen, Carlsbad, CA), and the sequence was verified by dye terminator chemistry on an ABI 377, 373, or 370 genetic analyzer (Applied Biosystems, Foster City, CA). Murine IL-17C was found by homology tBLASTn search of the GenBank High Throughput Genome Sequence database using the human ortholog. The full-length gene was predicted by homology comparison and gene specific forward and reverse primers were designed from this predicted sequence. The 5′ primer TGCTGCCATG GCCACCGTCACCGTCA and 3′ primer CACTGTGTAGACCTGGGA AGAACGCACGT were used to do touch-down PCR using a murine T cell transfer inflammatory bowel disease library.

IL-17D. GenBank at database entry gi434047 was identified in a computational screen by homology to IL-17 family members. PCR primers were designed based on this sequence and used to clone a partial cDNA from a Marathon ready fetal spleen library (Clontech Laboratories, Palo Alto, CA). The sequence of this fragment identified additional GenBank EST containing the missing 5′ sequence and the primers ACCTCGCTC AGTCGGAAGCTTATGTTGGGGGCACTGGTCTGGATGCTGGTAGC CGGCTTCCTGCTGGCGC and GGGGCAGGACCGGCCTCAGGGGC CAGC were used to complete the full-length cloning. Using the full-length human sequence, a rat EST (AI230670) was identified containing the rodent IL-17D leader region. Marathon ready mouse cDNA libraries (Clontech Laboratories) were amplified with primers ATGTTGGGGACACTG GTCTGGATGCTCCTCGTCGGCTTCCT and GGACCTGATGCATGC AGGAAGCTGCGC to obtain the full-length mouse cDNA. PCR fragments were cloned into the TOPO vector (Invitrogen) and the sequence was verified as above.

IL-17F. The IL-17F sequence was identified in the Human Genome Sciences EST database and the cDNA clone (HTXOR44) was supplied by Human Genome Sciences. The sequence was confirmed and completed as above. Murine IL-17F was found by homology BLASTn search of the Ensembl mouse genomic sequences with its human orthologue. The full-length gene was predicted from this genomic sequence and forward and reverse primers were designed. The 5′ primer ATGGTCAAGTCTTTGC TACTGTTGATGTT and 3′ primer TCAGGCCGCTTGGTGGACAAT GGGCT were used to do PCR using a mouse Th2 library.

IL-25. Human IL-25 cDNA was amplified from a human dendritic cell library using mouse IL-25 gene specific primers in a vector-anchored nested PCR. Based on the sequence of this fragment, primers ATGTAC CAGGTGGTTCCATTCTTG and CTAAGCCATGACCCGGGGCCGCA CACACACAC were used to amplify the cDNA, followed by cloning into TOPO vector and sequence confirmation.

Recombinant Ad and protein production

The full-length cDNAs for IL-17 family members were subcloned into the Ad transfer vector. The vector and recombinant Ad production were as described (14). 293 cells (5×10^8) (Quantum Biotechnologies, Montreal, Canada) were infected with a multiplicity of infection of ~10 Ad-mouse (m) IL-25 in 1 L culture media formulation 1 medium (CellWorks, San Diego, CA) and incubated for 5 days in a cell factory (Nalge Nunc International, Naperville, IL). Culture medium was dialyzed (membrane tubing, m.w. 6000–8000; Spectrum Laboratories, Rancho Dominguez, CA) vs 50 mM Tris-HCl, pH 8.0, 1 mM EDTA (Buffer A and passed-over HiTrap Q; Pharmacia, Uppsala, Sweden) to remove virus and many contaminating proteins.

Animals

Female BALB/cAnN, 129, 129.RAG2KO, and 129.γcKO-RAG2KO were obtained from Taconic Farms (Germantown, NY). IL-4KO (15), B6.SJL-ptprc^a/BoAiTac-B2 m (β_2MKO) RAG2KO, and NK1.1 congenic (16) mice on the BALB/c background were maintained at DNAX (Palo Alto, CA). 129Sv/Ev-IL-13KO (IL-13KO) mice were generated and maintained at the DNAX Research Institute (17); WBB6F1/J-Kit^W/Kit^{W-} (WW/kit), their congenic normal littermates (W/W$^+$), B6D2F1/J, and C57BL/6 were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were between 5 and 7 wk of age at the beginning of each experiment and were housed under specific pathogen-free conditions at DNAX.

Nasal administrations

Mice were anesthetized lightly with isofluorane and given 1×10^{10} Ad particles in 50 μl of saline intranasally (i.n.). For i.n. administration of recombinant protein, anesthetized mice were given 5 μg of IL-25 in 50 μl saline. Mice were held upright until breathing was steady.

Bronchiolar lavage fluid (BAL) and lung tissue collection

At specific time points following protein or Ad administration, mice were euthanized and the BAL was harvested via the trachea by flushing with 1 ml of RPMI 1640. Aliquots of the BAL fluid were cytospun onto glass slides, stained with Wright-Giemsa (Sigma-Aldrich, St. Louis, MO) and evaluated for cell types. Data was analyzed using a statistical program, InstatP (GraphPad, San Diego, CA), and numbers of cells were calculated as mean and SEM. In other experiments, the lungs were excised and snap frozen with liquid nitrogen and stored at −80°C until processing for RNA analysis or fixed in formalin and processed for histological staining with H&E and periodic acid-Schiff (PAS) stains (Idexx, West Sacramento, CA).

Infection models

Mice were infected with *Aspergillus fumigatus* (American Type Culture Collection 201795 (Manassas, VA); 13-day-old cultures grown at room temperature on malt extract agar) in an inhalation chamber using a 30-s exposure as previously described (18, 19). *Nippostrongylus brasiliensis* larvae were prepared at DNAX and delivered to mice as previously described (20). Briefly, 500 stage 3 larvae were injected s.c. into mice. Mice were sacrificed at the indicated timepoints and their small bowels were excised, flushed of fecal contents with ice-cold PBS, and snap-frozen. Samples were stored at −80°C until processing as described above.

Airway hyperreactivity

Male B6D2F1/J mice were anesthetized lightly and 5 μg of either mIL-25 protein or control protein (BSA) were delivered via the nares daily for 5 days. Mice were then tested for airway hyperresponsiveness to metacholine by the forced oscillation technique as previously described (21). A Student's *t* test was used to determine statistical significance between groups, with $p < 0.5$ being considered significant.

Antibodies

For cell depletions in vivo, mice were given 1 mg of mAb 1 day before and 2 days after injection of recombinant protein or Ad. Abs used for depletion included anti-Ly-6G (RB6–8C5) and anti-NK1.1 (PK136) (22). Anti-IL-5 mAb (TRFK5) was used as described above for cell-depleting mAb. mAb used for FACS analysis included anti-mouse Ly-6G, Thy-1, and CD45R/B220. Anti-mouse IL-5-PE mAb was used for intracellular staining as previously described (23). All FACS mAb were obtained from BD PharMingen (San Diego, CA) and were used according to the manufacturer's instructions.

Quantitation of cytokine transcripts by real-time PCR

Frozen lung tissue was homogenized and total RNA was extracted using Maxi-prep RNeasy columns according to the manufacturer's instructions and stored at −80. For RT-PCR, RNA was incubated with 10 U of DNase I (Boehringer Mannheim, Indianapolis, IN) in the presence of RNasin (Promega, Madison, WI) for 30 min at 37°C. The samples were then heat-inactivated at 70°C for 10 min, chilled, and reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) with random hexamers and poly(dT) oligos according to the manufacturer's protocol. Equivalent amounts of individual cDNA reactions from similarly treated mice (six to eight mice per timepoint) were combined to create pooled samples. Primers

were either obtained from PerkinElmer (Foster City, CA) or generated with Primer Express software (PerkinElmer) and were synthesized by us. Whenever possible, primer pairs were designed to span intron/exon borders. PCR were performed at 95°C for 15 s followed by 60°C for 1 min using an ABI Geneamp 5700 sequence detection system and SYBR green buffer according to the manufacturer (PerkinElmer). PCR amplification of the housekeeping gene ubiquitin was performed for each sample to control for sample loading and to allow normalization between samples according to the manufacturer's instructions (PerkinElmer). Both water and genomic DNA controls were included to insure specificity. Each data point was evaluated for integrity by analysis of the amplification plot and dissassociation curves. The ubiquitin normalized data was expressed as the fold induction of gene expression in treated mice compared with that in untreated mice.

Results

Identification and structural motif analysis of new IL-17 family members

To identify novel IL-17 homologues, we performed a position-specific iterated-BLAST search against the GenBank NR protein database, selected significant hits for iterative searching, and built an IL-17 position-specific scoring matrix (PSSM). This PSSM was used to identify additional family members from various proprietary sequence databases, expanding the IL-17 family to include six members. The alignment of these sequences shows a highly

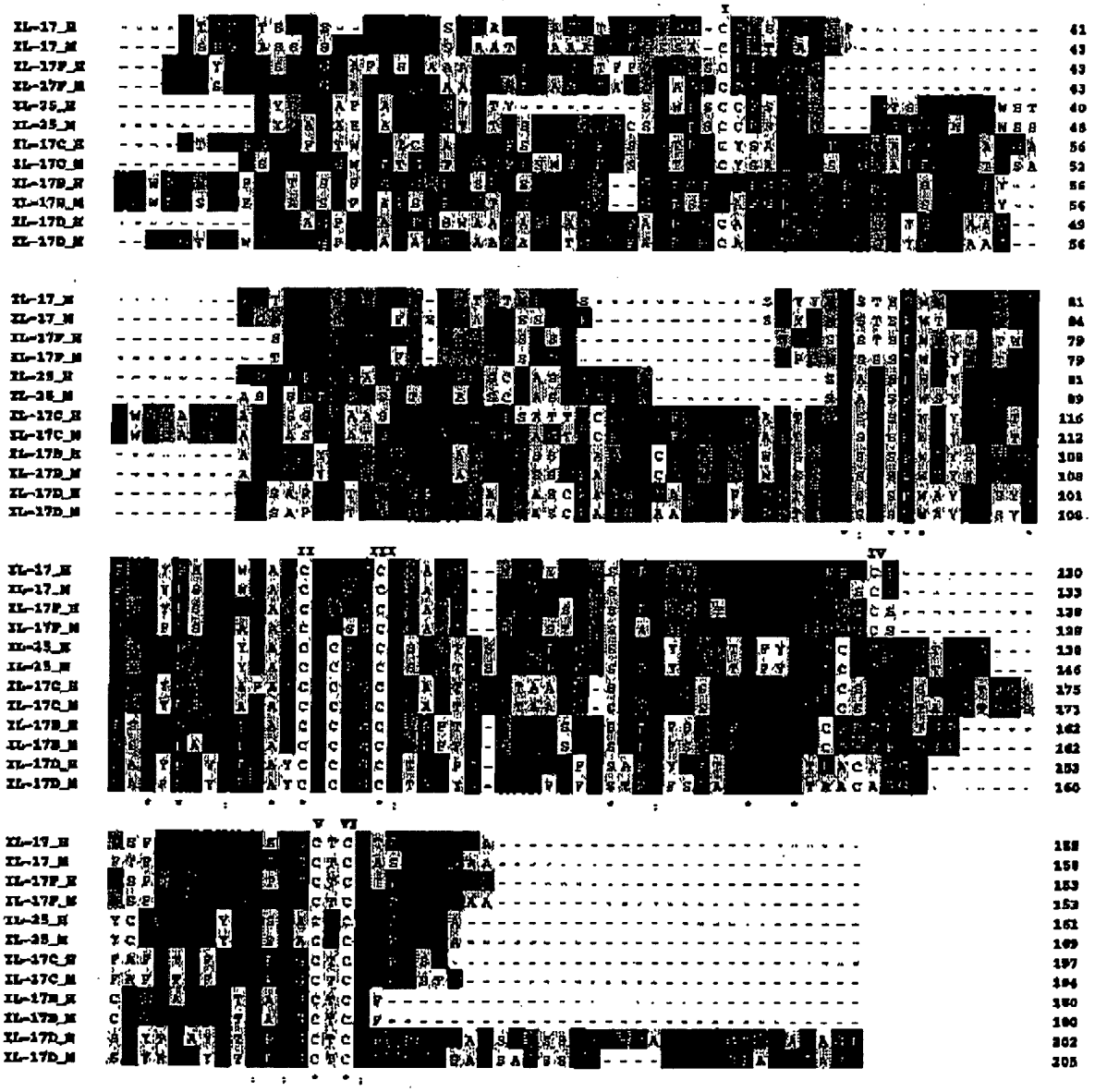

FIGURE 1. Alignment of amino acid sequence of IL-17 family members. Mature IL-17 family member protein sequences were aligned using ClustalX and hand adjustment. An amino acid coloring scheme correlates chemically similar residues as follows: green (hydrophobic), red (acidic), blue (basic), yellow (C), orange (aromatic), black (structure breaking), purple (amido), and gray (small). Conserved cysteine-knot residues I-VI are labeled accordingly. The predicted N-linked glycosylation site is marked with a caret (^). Conserved and identical residues are marked by a colon (:) and an asterisk (*), respectively.

variable N-terminal region with 1–4 cysteine residues present (Fig. 1). The C-terminal sequence of all IL-17 family members contains a set of five spatially conserved cysteine residues. However, the total number of cysteine residues varies considerably within the family: mouse and human IL-25 have 10 and 11 cysteines, respectively, while IL-17 and IL-17F have 6. The expanded IL-17 family PSSM was used to search the Protein Data Bank sequence database, where a weak match to nerve growth factor was identified (data not shown). The conserved C-terminal CXC motif and four additional cysteine residues with relative spatial conservation suggest that IL-17 may be related to the so-called cysteine-knot structural superfamily (24). Among the cysteine-knot growth factors, negligible sequence identity is seen outside the core knot structure. The formation of dimers among IL-17 family members is a common theme seen among the cysteine-knot growth factors. In fact, recent findings demonstrated that IL-17F belongs to the cysteine-knot growth factor family (25).

Adenoviral expression of IL-17, IL-17C, and IL-17F produces neutrophilia, while IL-25 produces eosinophilia in the lung

Demonstration of function for novel genes that have been identified by bioinformatics is a critical step in functional genomics. The use of Ad constructs to ectopically express unknown genes in the lung epithelium has proven a useful strategy for the expression and functional evaluation of novel molecules in vivo (12). We selected mIL-17, human (h) IL-17C, hIL-17F, hIL-25, and mIL-25 from among the new IL-17 family members described in Fig. 1 for further analysis. Mice were given 1×10^{10} particles of recombinant Ad i.n., and BAL and lung tissue were harvested at day 7 following infection. Mice given control Ad developed mild neutrophilia in the BAL fluid at day 7 consistent with the mild inflammatory response expected from nonreplicating Ad (Fig. 2*A*). However, BAL fluid from mIL-17, hIL-17C, and IL-17F Ad-infected mice contained far more neutrophils than were present in control Ad-infected mice. In contrast, both human and mouse IL-25-infected mice showed large numbers of BAL fluid eosinophils compared with controls. Human and mouse IL-25 Ad infections produced similar levels of neutrophils and eosinophils, demonstrating the species cross-reactivity of human IL-25.

To confirm that the eosinophil response was due to ectopically expressed adenoviral IL-25, we gave mice mIL-25 protein purified from 293 cell supernatants infected with mIL-25 Ad. Mice given 5 μg of purified IL-25 protein i.n. produced eosinophilia at day 7 without the neutrophilia observed with IL-25 Ad (Fig. 2*B*). Additional experiments to further characterize this response showed that as little as 0.5 μg of IL-25 protein i.n. resulted in lung eosinophilia and that, regardless of the amount of IL-25 given, a minimum of three days following IL-25 protein administration was required for this result (data not shown). These results suggested that IL-25 promoted eosinophil development and not simply eosinophil recruitment from the blood. Interestingly, expression of IL-25 by Ad caused neutrophil infiltrate as well as eosinophilia, while administration of mIL-25 protein resulted in a distinct eosinophilic response. This suggested that IL-25 may support neutrophil recruitment in the context of adenoviral infection.

IL-17F expression resulted in Th1, while IL-25 produced Th2-like inflammatory gene expression

We have developed a novel approach for evaluating the function of cytokine-like genes following either gene transfer or administration of recombinant protein in vivo. This approach uses 96-well real-time PCR primer arrays to measure changes in mRNA levels for a wide range of cytokines, chemokines, and chemokine receptors. Following the identification of cellular infiltrate in the BAL of mice infected with novel gene Ad, we asked whether gene expression profiling would provide further insight into the biological activities of the new proteins. We selected one of the neutrophilia-producing family members, hIL-17F, to compare and contrast with the eosinophilia-producing IL-25. At day 7 following infection, lung tissue from mice given hIL-17F Ad showed substantial increases in the mRNA for inflammatory cytokines and chemokines, including IL-6, IFN-γ, inflammatory protein 10, and monokine

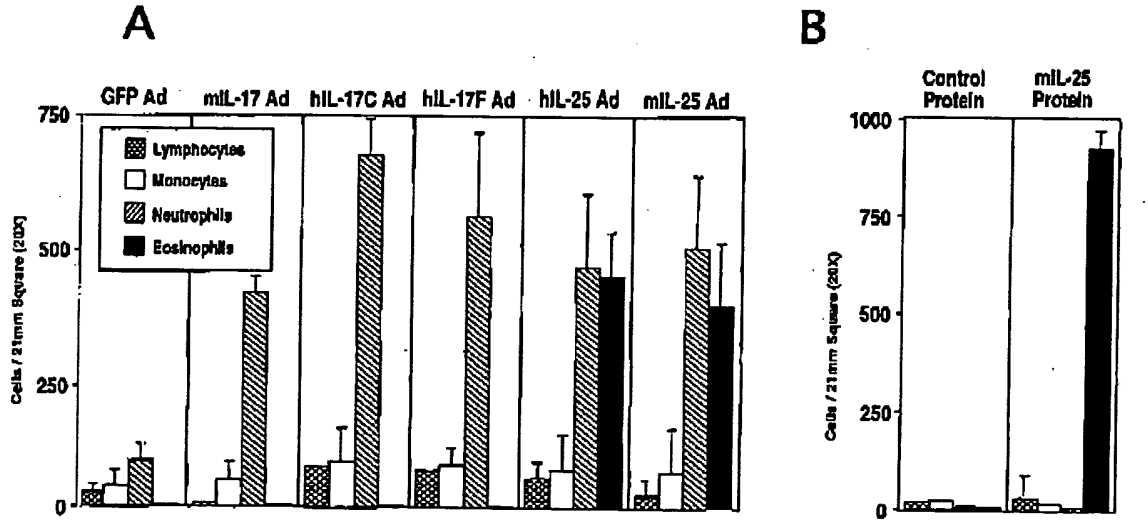

FIGURE 2. Lung administration of IL-17, IL-17C, and IL-17F produced neutrophilia while IL-25 produced eosinophilia in the BAL. *A*, BALB/cAnN mice ($n = 5$) were given 1×10^{10} adenoviral particles (Ad) i.n. *B*, Recombinant control protein or mIL-25 protein (5 μg) was given to mice ($n = 5$) i.n. Seven days post-infection or post-protein administration, mice were sacrificed and BAL fluid was harvested. BAL cells were cytospun, stained with Wright-Giemsa and relative cell types were determined microscopically with a grid-marked eyepiece. Data shown are the relative cell types within the grid square. Results shown are representative of over five experiments.

induced by IFN-γ (Fig. 3). The stimulation of this group of inflammatory genes may predict the influx of neutrophils in the BAL of mice infected with IL-17F Ad.

In contrast, expression of mIL-25 via Ad in the lung produced a Th2-like response with elevations in mRNA for IL-4, IL-5, and IL-13 and the chemokines LIX, TARC, and eotaxin. Similar to the

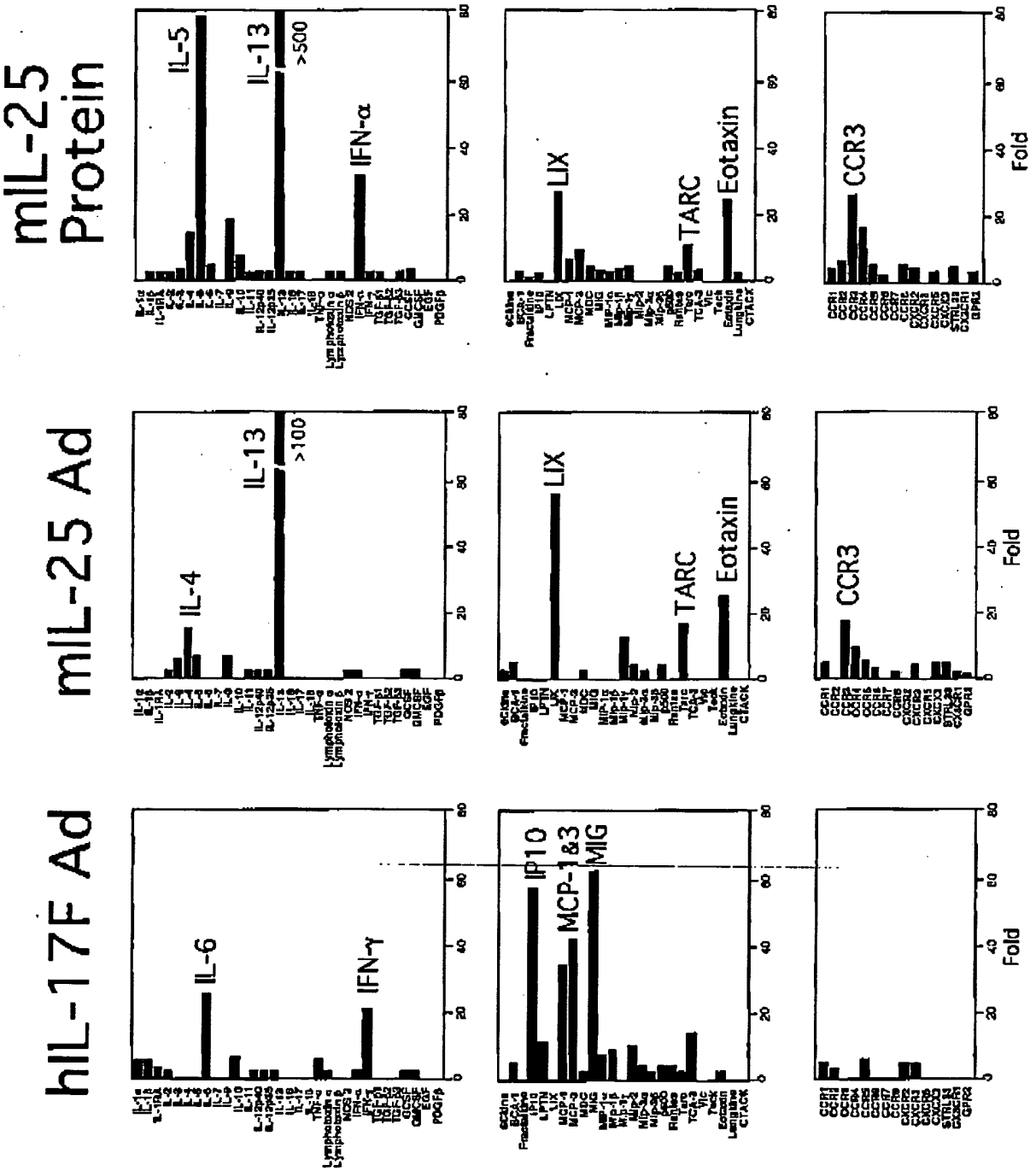

FIGURE 3. IL-17F expressed in the lung up-regulated Th1 response genes, while IL-25 up-regulated Th2 response genes. BALB/cAnN mice were given either 1×10^{10} particles of control, hIL-17F, or mIL-25 Ad, or 5 μg of recombinant control or mIL-25 protein i.n. Seven days later, mice were sacrificed and lungs were harvested, snap-frozen, and processed for RNA. Reverse-transcribed samples were analyzed on 96-well plate arrays of Taqman PCR primers for cytokines, chemokines, or chemokine receptors as described in *Materials and Methods*. Data shown is the fold increase over the control group of ubiquitin-normalized values. Data is representative of over five experiments.

results observed with IL-25 Ad, mice given purified IL-25 protein produced high levels of IL-5 and even higher levels of IL-13, suggesting that these cytokines may have been somewhat inhibited by the concurrent response to adenoviral infection. It is interesting to note that while both IL-5 (80-fold) and IL-13 (>500-fold) were stimulated to a greater extent than IL-4 (20-fold) by IL-25 administration, mice given either mIL-25 Ad or protein also showed substantially increased levels of CCR3, most likely as a result of infiltrating eosinophils. As assessed by the responses in mouse lung, the biological activity of IL-25 differed markedly from the other IL-17-related molecules and the phenotype suggested that IL-25 may play a role in the establishment of Th2-like responses in vivo.

Mice given IL-25 develop cellular infiltrate and mucus secretion in the lung

To further characterize the response to IL-25, we gave mice one dose of 5 μg mIL-25 protein i.n. and harvested lung tissue at daily intervals for 7 days to determine the time course of IL-25-induced pathologies. IL-5 and IL-13 mRNA were rapidly induced in lung tissue by IL-25, with peak levels at 72-h postadministration. Interestingly, normalized expression of IL-13 was much higher throughout the time course than IL-5. Examination of lung histology after i.n. IL-25 protein administration showed that peak IL-5 and IL-13 mRNA induction was followed by cellular infiltrate, mucus production, and epithelial cell hyperplasia (Fig. 4). Low, but marked, cellular infiltrate was clearly present in the H&E-stained section from day 3 post-IL-25 protein treatment mice. This infiltration increased through day 7 with cells ultimately identifiable in the lumenal space. High-power examination showed that the majority of these cells were eosinophils, with some monocytes and lymphocytes (data not shown). PAS staining identified production of mucus by epithelial cells as early as day 3 posttreatment while complete airway occlusion with PAS staining mucus is evident at day 7. These results demonstrate that IL-25 is capable of inducing the hallmark components of allergic airway disease, including IL-4, IL-5, and IL-13 expression, cellular infiltrate, and mucus production.

Mice given IL-25 develop airway hyperreactivity

Because the responses described above frequently correlate with development of airway hyperreactivity, we investigated whether administration of IL-25 protein alone to naive mice would be sufficient to induce airway hyperresponsiveness to methacholine. Mice given IL-25 protein i.n. daily for 5 days developed hyperreactivity when challenged with methacholine in vivo (Fig. 5). However, a single dose of IL-25, which produced IL-5, IL-13, and mucus production, was not sufficient to induce hyperreactivity (data not shown), corroborating previous work showing that a chronic regimen of allergic symptom induction is required to develop airway hyperreactivity (26, 27). Together, these results demonstrate that exposure of the murine airway to purified IL-25 protein alone is sufficient to promote both the pathological and physiological features of an allergic response.

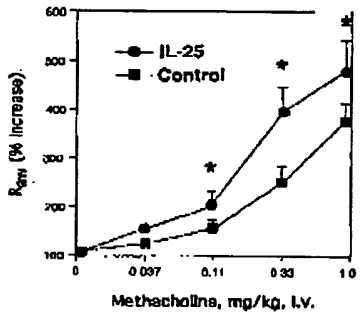

FIGURE 5. Lung administration of IL-25 caused airway hyperreactivity. B6D2F1/J mice were given 5 μg of IL-25 or control (BSA) protein daily for 5 days. On day 5, mice were assayed of hyperreactivity by methacholine challenge as described in *Materials and Methods*. Data shown is the R_{aw} (% increase) for increasing dosages of methacholine. *, $p < 0.05$.

IL-25 mRNA is increased during infection in the lung and gut

To understand the regulation and disease association of IL-25, we tested a number of infection and immune response models from a variety of murine tissue sources. Among these samples, IL-25 message was up-regulated in the lung following *A. fumigatus* infection, and in the gut following *N. brasiliensis* infection. In lung tissue (Fig. 6), IL-25 message was up-regulated ~10-fold following aerosolized infection with live *A. fumigatus* spores. This up-regulation was maximal at 48-h postinfection, and returned to baseline levels by day 7 postinfection. Fig. 6 also shows the time course of IL-25 expression in the small bowel following *N. brasiliensis* infection. Expression was up-regulated ~6-fold between

FIGURE 4. Lung administration of IL-25 caused eosinophilic infiltrate and mucus production. BALB/cAnN mice were given 5 μg of mIL-25 protein i.n. At timepoints from days 0 to 7 post-IL-25 administration, mice were sacrificed, and lungs were harvested and assayed for cytokine production or fixed and stained for mucus and cellular infiltrate. Data shown are H&E- and PAS-stained lung sections. Solid arrowheads indicate cellular infiltrate. Open arrowheads indicate mucus accumulation.

days 7 and 13 postinfection, and decreased through day 13. This longer time course closely matches the arrival of worms into the gastrointestinal tracts of the infected mice (28). In both systems, however, the normalized expression of IL-25 message was in the range of other low expression, high potency Th2 cytokines such as IL-4 and thus may preclude detection by histological means. However, despite this low level of expression, the active up-regulation of IL-25 mRNA in tissues responding to these pathogens suggests that this cytokine may have a role in the Th2 differentiation of the immune response to fungi and parasites.

IL-25 generates eosinophilia via IL-5 and IL-13

Known activities of IL-5 and IL-13 suggested that these cytokines were key components of the eosinophilic response following IL-25 administration in the lung described in Fig. 2 (29, 30). To confirm this, 5 μg of IL-25 protein was administered i.n. to BALB/cAnN, IL-4KO, IL-13KO, and anti-IL-5-treated mice. One week later, BAL fluid was harvested and evaluated for eosinophils by cytospin. Wild-type controls and IL-4KO mice showed dramatic eosinophilic responses to IL-25 protein administration, however, anti-IL-5 treated mice showed greatly reduced numbers of BAL eosinophils and IL-13KO produced no eosinophilia in response to IL-25 (Fig. 7). These results demonstrated that the eosinophilia in the lungs of mice given IL-25 required both IL-13 and IL-5 as intermediates

Lymphocytes, NK cells, mast cells, basophils, and granulocytes are not required for IL-25 responsiveness

The rapid induction of IL-5 and IL-13 message following IL-25 exposure suggested that the responding cell type is resident in the lung. To identify cells responding to IL-25, recombinant cytokine was given i.n. to mice made deficient in specific cell types by mAb depletion or genetic deletions (Fig. 8*A*). Strikingly, RAG2KO mice, deficient in both T and B cells, produced high numbers of lung eosinophils, demonstrating that lymphocytes were not required for this response. The induction of IL-5, IL-13, and eotaxin mRNA by IL-25 i.n. administration was also similar in control and RAG2KO mice, demonstrating that lymphocytes were not the main source of

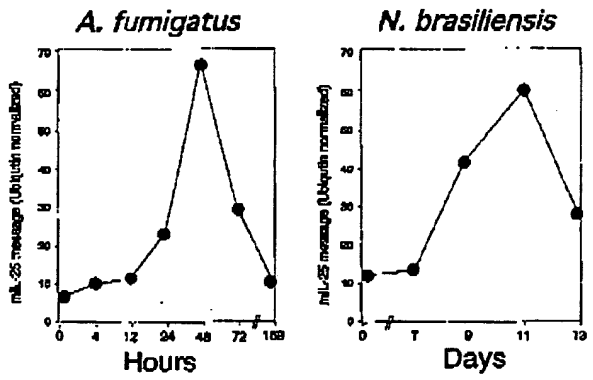

FIGURE 6. IL-25 is expressed in the lung and gut during pathogen infections. BALB/cAnN mice were infected with either (*A*) *A. fumigatus* spores via inhalation or (*B*) *N. brasiliensis* larvae via s.c. injection as described in *Materials and Methods*. At timepoints following infection, mice were sacrificed and their lungs or small bowel were harvested, snap-frozen, and processed for RNA as described in *Materials and Methods*. IL-25 RNA levels were assayed by real-time PCR and normalized to ubiquitin RNA levels for each timepoint. Data shown are the ubiquitin-normalized values of mIL-25 RNA at each timepoint. Results are representative of two experiments.

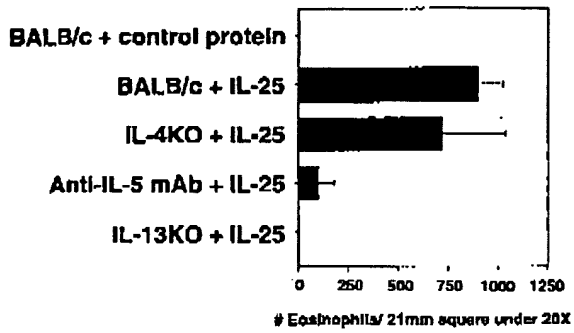

FIGURE 7. IL-25-mediated eosinophilia requires IL-13. BALB/cAnN, IL-4KO, and IL-13KO mice were given IL-25 protein (5 μg) i.n. Additional BALB/cAnN mice were given 1 mg of anti-IL-5 mAb on day −1 and on day 2 after IL-25 protein administration; $n = 5$ for each treatment group. Seven days later, mice were sacrificed and BAL fluid was harvested and evaluated as described in Fig. 2. Data shown are the eosinophils in the BAL for each treatment group. Results shown are representative of three experiments.

these cytokines (data not shown). Furthermore, the depletion of NK1.1 cells with PK136 mAb did not prevent the development of eosinophilia in the lungs of mice given mIL-25 protein. Mast cells are another known source of IL-5 and IL-13, however, eosinophilia was

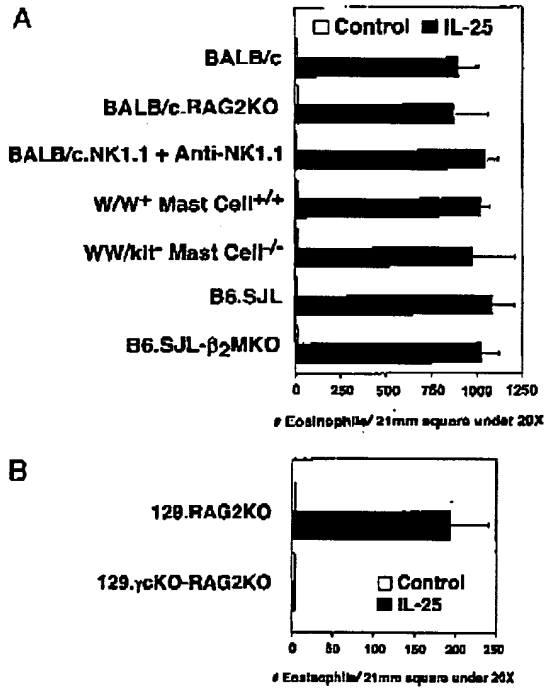

FIGURE 8. IL-25 promotes eosinophilia in the absence of lymphocytes, NK cells, and mast cells. *A*, BALB/cAnN mice and mice deficient in lymphocytes (RAG2KO), $CD8^+$ T cells (β_2MKO), mast cells (WW/kit), or NK cells (anti-NK1.1-treated) were given control or mIL-25 protein on day 0. Seven days later, lung BAL fluid was assayed as described in Fig. 2. *B*, 129.RAG2KO and 129.γcKO-RAG2KO mice were given control or mIL-25 protein on day 0 and assayed on day 7 as described above; $n = 5$ mice per treatment group. Data shown are representative of three experiments.

similar between mast cell-deficient (WBB6F1/J-Kit^{W}/Kit^{W-}) and control mice given IL-25 protein. Finally, β_2MKO mice with neither NK1$^+$ CD4$^+$ T nor CD8$^+$ T cells responded to IL-25 by producing similar numbers of BAL eosinophils as did control mice. In all of the above experiments, groups of treated mice given control 293 cell supernatant i.n. did not develop eosinophilia (Fig. 8 and data not shown). Recently, it has been shown that both basophils and mast cells produce IL-5 and IL-13 in response to IL-18 (31). We cultured sorted mast cells and basophils with IL-25, IL-18, or IL-25 + IL-18 and detected both IL-5 and IL-13 from IL-18 cultured cell supernatants. However, no IL-5 or IL-13 could be detected from IL-25-cultured cells, nor was there any indication of synergy in IL-5 or IL-13 production from cells cultured with both IL-18 and IL-25 (data not shown). Taken together, these in vivo and in vitro results suggested that neither mast cells nor basophils were a significant source of IL-5 or IL-13 in response to IL-25.

In addition to testing the IL-25 responsiveness of BALB/c RAG2KO mice, we tested the responsiveness of 129.RAG2KO and 129.γcKO-RAG2KO mice. Fig. 8*B* shows that eosinophilia was strongly induced in the 129 background RAG2KO mice, although these levels were lower than in the BALB/cAnN background RAG2KO strain. Interestingly, 129.γcKO-RAG2KO mice did not respond to IL-25 given i.n.

IL-25 induces IL-5 from a hemopoietically derived cell type

In a second approach to identifying the cells producing IL-25-induced IL-5 and IL-13, lung cells from mice given mIL-25 protein i.n. were activated in vitro with PMA/Iono and brefeldin A and stained for intracellular cytokine and various lineage-specific cell surface markers. This approach initially revealed a cell population that was slightly larger and more granular than lymphocytes and was positive for intracellular IL-5 (Fig. 9*A*). This distinct population constituted of 14% of the cells in gate 1 and represented ~1–2% of the total cells in the lung. Importantly, no IL-5-staining cells could be detected from lung cells of mice given control protein i.n. even though these cells had been activated in vitro identically to those from IL-25-treated mice.

We also gave IL-25 to BALB/cAnN background RAG2KO mice to determine whether the BAL eosinophilia observed in these mice (in Fig. 7*A*) was accompanied by IL-5-producing cells. In BALB/cAnN mice most of the cells that stained for intracellular IL-5 were CD4$^-$, although some CD4$^+$ cells were detected. In contrast, RAG2KO mice contained cells that stained for intracellular IL-5 but were not CD4$^+$. This finding confirmed that the cell population responding to IL-25 in the lung need not be lymphocytic in origin. Further analysis showed that the IL-5-producing cells in RAG2KO mice are low to negative for Thy-1 and CD45R/B220, and negative for c-*kit*, Ly6G, Ly49, CD3, CD4, γδ TCR, and intracellular CD3ε (Fig. 9*B* and data not shown). In addition, depletion of Ly6G$^+$ granulocytes in vivo with RB6–8C5 mAb did not deplete IL-5-positive cells in the lung by FACS (data not shown). Together these results confirmed the results in Fig. 7 that the cell type responsible for IL-5 and, most likely, IL-13 production following IL-25 exposure is of a nonlymphocyte,

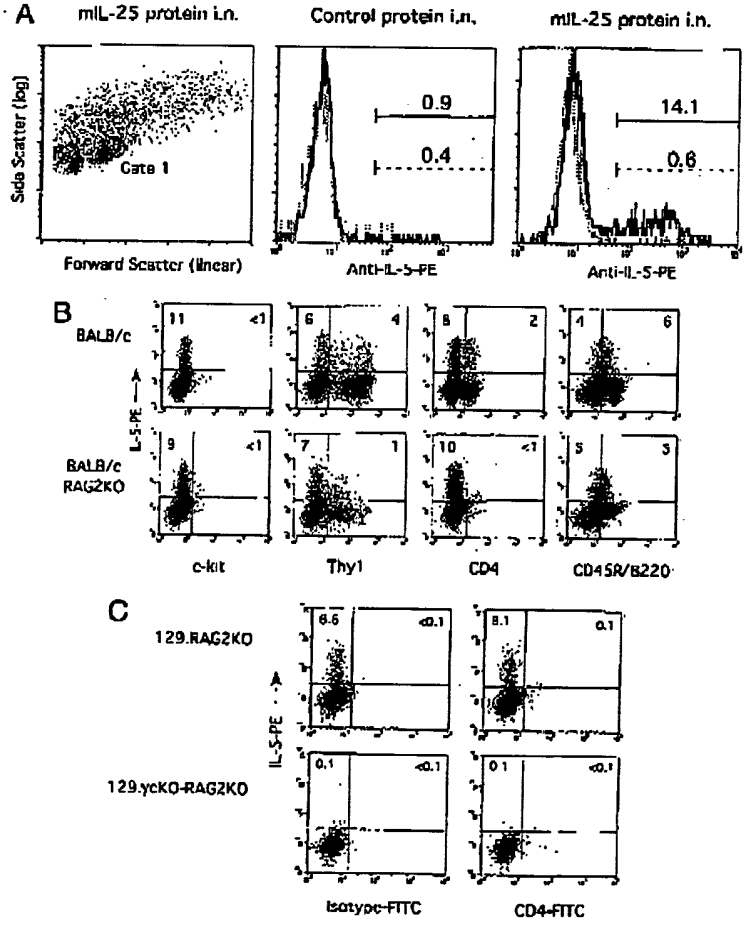

FIGURE 9. IL-25 induced IL-5 production from a distinct cell type in the lung. Mice were given 5 μg of control or mIL-25 i.n. on day 0 and sacrificed on day 7. Lungs were harvested, mechanically dissociated into a single cell suspension, and incubated with PMA/Iono and brefeldin A as described in *Materials and Methods*. Cells were stained for intracellular IL-5 or control Ig and analyzed by FACS. *A*, Lung cells from control protein or mIL-25 protein-treated mice were gated by forward and side scatter (Gate1) and intracellular IL-5-positive cells were displayed (solid line). Isotype Ig control is also shown (broken line). *B*, BALB/cAnN and BALB/cAnN-RAG2KO mice were given 5 μg of mIL-25 protein i.n. on day 0 and analyzed on day 7 as described above. *C*, 129.RAG2KO and 129.γcKO-RAG2KO mice were given 5 μg of mIL-25 protein i.n. and analyzed as described above. Quadrants were set by staining of control isotype Ig. Surface stains were performed before cell permeabilization. Data shown is representative of at least three experiments.

non-NK, nongranulocyte lineage. Finally, we stained cells from 129.RAG2KO and 129.γcKO-RAG2KO mice to determine whether the unresponsiveness of γc-chain KO mice observed in Fig. 7*B* was due to the absence of a responding cell population. Fig. 9*C* shows that cells from lungs of 129.RAG2KO mice given IL-25 i.n. clearly stained positive for intracellular IL-5, whereas lung tissue from 129.γcKO-RAG2KO mice given IL-25 i.n. did not produce cells that stained for intracellular IL-5. This finding suggested that the absence of eosinophilia observed in this mouse strain in Fig. 8 was due to the absence of an IL-5-producing cell type and not due to a defect in the eosinophil lineage. Future experiments involving more complex depletions and in vitro reconstitutions may ultimately identify this distinct cell subset.

Discussion

In this report, we describe a novel approach for the analysis of biological activity of new molecules discovered by homology to known cytokines. Using this approach, we show that IL-17, and two newly described members of the IL-17 family, IL-17C and IL-17F, functionally resemble one another in their potential to induce inflammatory genes such as IFN-γ and IL-6 and produce neutrophilia when expressed in the lung. In contrast, new IL-17 family member IL-25 was found to promote the expression of prototypical Th2 genes such as *IL-4*, *IL-5*, *IL-13*, and eotaxin and produce lung eosinophilia. Following a single i.n. dose of IL-25 protein, expression of IL-5 and IL-13 peaked at day 3. Accompanying the expression of these Th2 cytokines, cellular infiltrate, thickening of airway tissue, and mucus production were readily identifiable through day 7 postadministration. Mice given IL-25 recombinant protein also developed airway hyperreactivity, suggesting that the cellular infiltrate and mucus production observed developed into physiological airway pathology. The finding that IL-25 can promote mucus production and airway hyperreactivity is not altogether unexpected given the clear ability of IL-13 to promote these pathologies (32, 33). Taken together, however, these results show that the production of IL-25 in the lung can result in all of the prototypical hallmarks of Th2-mediated airway disease, involving infiltrate, cytokine production, tissue reorganization, mucus secretion and airway hyperreactivity. This acute lung response to IL-25 is not mediated by Th2 cells, however, as T cell-deficient RAG2KO mice respond to IL-25 as well as control mice. Additional work by members of our group has shown that mice given systemic IL-25 had increased levels of serum IgE, IgG1 and IgA levels, as well as blood eosinophilia. These IL-25-treated mice also developed digestive tract pathologies including epithelial cell hyperplasia, mucus production, and eosinophilic infiltrate (14).

Initially it may seem confusing that molecules with such high sequence homology display such different biological properties. However, a close examination of the IL-17 family sequences shows a conserved cysteine-knot structure with considerable sequence divergence at the N terminus. Primary sequence homology also differs between family members with hIL-17 and hIL-17F having the highest homology (44%), while hIL-17 and hIL-25 have the lowest (15%). In addition to primary sequence, a preliminary relationship between biological function and structure emerges in that IL-17 family members with few cysteines, i.e., IL-17 and IL-17F (both with six) produce Th1-like inflammation while family members with the greatest number of cysteines, i.e., human and mouse IL-25 (10 and 11, respectively), produced Th2-like responses. These findings suggest that a combination of N-terminal region diversity, primary sequence, and cysteine-dependent motifs may be responsible for specific interaction of these molecules with their cognate signaling receptors and thus divergent biological effects. Using a similar bioinformatics approach, we have expanded the IL-17R family to include five related type I membrane proteins (D. M. Gorman, unpublished data). Although the final identification of these IL-17-related receptors to their respective ligands should help reveal the biological differences seen among these related cytokines, at this time we believe that the divergence between IL-25 and the other IL-17 family molecules will prove to be receptor/signaling pathway-mediated and not via other mechanisms.

To determine whether IL-25 might play a role in pathogenic responses, IL-25 mRNA was measured in lung and gut in several models in mice. The expression of IL-25 mRNA was increased during fungal infection of the lung with *A. fumigatus* and helminth infection of the gut with *N. brasiliensis* while total normalized expression remained low. This suggested that, while highly potent, either IL-25 or its cell source may be rare, or both. Future experiments using IL-25 blocking Abs or IL-25KO mice may help to define the relationship between IL-25 expression and pathology in these models. Interestingly, no detectable increase in IL-25 mRNA was detected in lung tissue from mice which had been sensitized and aerosol challenged with hen egg OVA (data not shown). A better understanding of the role of IL-25, its cell source and its variable expression in these in vivo model systems should provide points of future intervention for allergic and infectious disease at the epithelial border.

Further analysis of mice given IL-25 protein i.n. demonstrated that IL-5 and IL-13 were critical mediators in the development of pathology. Treatment of mice with a neutralizing anti-IL-5 mAb prevented most of the eosinophilia observed in IL-25 control-treated mice. Our interpretation of this result is that IL-5 may be required for the generation of new eosinophils and that treatment of mice with anti-IL-5 blocked this response, but not the production of IL-4 and IL-13, from these mice. The low numbers of eosinophils observed in the lungs of anti-IL-5-treated mice likely reflected the recruitment of pre-existing eosinophils to the lung. Additional experiments demonstrated that IL-25 protein did not have any eosinophil chemoattractant ability in vitro (data not shown), suggesting that the lung eosinophilia following IL-25 administration may have involved IL-13-induced genes such as eotaxin (34, 35) and *VCAM-1* (36). Previous reports have suggested that IL-13 is required for lung eosinophilia and our findings suggested that IL-25 likely caused eosinophils via this previously described mechanism and not via another factor (32, 34, 35, 37).

Despite the typical Th2 cytokine profile induced by IL-25 in vivo, the principal cell type responding to IL-25 does not appear to be a T cell. The IL-5, IL-13-producing cell in the lung is present in comparable numbers in RAG2KO and control mice. Thus, the responding cell is neither a T nor B cell, although some $CD4^+$ IL-5-positive-staining cells were present in the lungs of IL-25-treated wild-type mice. In addition, the IL-5-producing cells appeared slightly larger and more granular than lymphocytes as judged by forward and side scatter. Double staining of RAG2KO lung cells for intracellular IL-5 and various markers showed low to negative expression of Thy-1 and CD45R/B220, but no detectable expression of c-*kit*, Ly6G, Ly49, CD3, CD4, γδ TCR, and intracellular CD3ε. Intracellular staining of IL-13 corroborated the IL-5 staining data and suggested that the same cell produced both IL-5 and IL-13 following IL-25 exposure. However, IL-13 staining was uniformly dimmer and may reflect the limitations of the anti-IL-13 mAb (data not shown). This unique surface staining profile eliminated most cell populations that might have been expected to produce IL-5 in vivo. IL-25 may act as a growth or differentiation factor for this cell type and this activity results in the accumulation

of IL-4-, IL-5-, and IL-13-producing cells at mucosal surfaces of the lung and gut.

We also investigated the response of RAG2KO and γcKO-RAG2KO following IL-25 administration. We showed that RAG2KO mice possessed a normal response to IL-25 by up-regulation of cells expressing IL-5. However, γcKO-RAG2KO mice did not respond to IL-25 administration by production of eosinophils nor did they produce IL-5-staining cells by FACS. Previous work has demonstrated that mice with targeted mutation in the common γ (γ_c)-chain locus are deficient in NK, $\gamma\delta$ T, and potentially other poorly characterized hemopoietic cell populations which require one of the receptors formed by the γ_c chain subunit for development (38–45). Our interpretation of our finding is that the cell type that normally produces IL-5 following exposure to IL-25 is missing due to the absence of the γ_c chain. This suggests that the source for these cells is hemopoietic, although they are not lymphocytes. Alternative mechanisms could involve a role for the γ_c chain in the IL-25R. However, preliminary receptor-ligand matching data suggests that the γ_c chain is not involved in the IL-25R (data not shown).

Finally, we report that the wide-screen analysis of downstream gene regulation by real-time PCR following administration of Ad or protein was effective in identifying the function of novel genes. Large-scale analysis of gene expression patterns has become an important analytical tool in many areas of biology. Most current approaches toward gene expression profiling of in vivo disease states involves the use of DNA microarray chips. Although this approach is appealing in its ability to interrogate thousands of gene transcripts for discovery of novel expression patterns, the technique is limited in sensitivity and by the quality of the annotations. For the functional analysis of gene products thought to participate in immune or inflammatory responses, we have found that RT-PCR measurements of changes in expression of a limited set of genes with known functions in immunity are much more useful than microarray analysis. RT-PCR with the Taqman system is ~100-fold more sensitive than DNA microarrays (S. D. Hurst and R. L. Coffman, unpublished data) permitting even rare, but potent, cytokines to be measured with accuracy. The patterns of change we have observed in a well-chosen panel of fewer than 100 genes have generally proven to predict the functional, structural, and cellular changes that are observed in the same samples, leading to clear, testable hypotheses about the activities of a novel gene. In contrast, DNA microarray analysis often failed to detect these expression changes or, more frequently, identified modulation of uninformative or unknown ESTs.

Demonstration of function in vivo is a critical, but often elusive, step in the evaluation of the biological role and therapeutic potential of genes identified by homology searching of sequence databases. The ongoing effort to identify new components and regulators of inflammation may ultimately result in future antagonist targets and drugs. Lung administration of recombinant Ad vectors expressing novel secreted genes is both efficient and informative enough to be used as a primary functional screen for cytokine-like molecules. Lung infection produces sustained local secretion of the protein in vivo for many days following infection. As demonstrated in this study, the nature of the infiltrating cells, changes in tissue structure, and alterations in gene expression profiles all give important information about the biological activity of the new molecule, in vivo.

Acknowledgments

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